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THE VAGAL CONTROL OF THE JEJUNAL AND ILEAL MOTILITY AND BLOOD FLOW

BY

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tic activity would inhibit intestinal motor activity. However, this classical concept of the extrinsic nervous control of the motility of the small intestine has been questioned by several investigators.

First, opinions diverge considerably regarding *the sympathetic innervation*. For instance, KURÉ, ICHIKO and ISHIKAWA (1931) concluded that the splanchnic nerves carry both excitatory and inhibitory fibres to the intestine. They stimulated the splanchnic nerves in dogs after the local application of nicotine to the coeliac ganglion in order to block the synapses between the pre- and postganglionic inhibitory sympathetic fibres to the small intestine. It was then observed that intestinal motility increased with the splanchnic stimulation and they ascribed this effect to an activation of spinal parasympathetic excitatory fibres reaching the splanchnic nerves via the dorsal thoracic roots. Their observations were confirmed by SEMBA and HIRAOKA (1957) but these investigators suggested that the motor responses could as well be due to an antidromic activation of afferent fibres in the splanchnic nerves, as excitatory intestinal responses could also be induced by antidromic stimulation of the thoracic dorsal roots. HUKUHARA (1934), however, failed to reproduce the experiments of KURÉ *et al.* and regarded the existence of the so called "spinal parasymphathetic nervous system" as highly doubtful.

CELANDER (1959), on the other hand, seriously questioned whether the splanchnic nerves at all convey any centrally controlled inhibitory fibres to the intestinal smooth muscles. At splanchnic nerve stimulation with physiological frequencies, i.e. below 8—10 impulses per second, he was always able to induce prompt and marked vasoconstrictor responses, while jejunal motility in these vagotomized, adrenalectomized cats showed only delayed inhibitory responses. If, however, small amounts of catechol amines were administered intravenously, profound inhibitions in motility were regularly induced, appearing as promptly as the vasoconstrictor effects. CELANDER pointed out that in practically all earlier studies the splanchnic nerves had been stimulated with supraphysiological frequencies, and presented evidence that an overflow of transmitter from the vasoconstrictor nerve endings might be responsible for the intestinal inhibitory responses to high frequency splanchnic nerve stimulation.

KOCK (1959) confirmed CELANDER's observations and showed that the jejunal inhibitions produced in acutely vagotomized cats by centrally mediated reflex increases of sympathetic activity were abolished when the catecholamine secretion into the blood stream by the adrenal medulla was interfered with. In contrast, neurogenically mediated intestinal inhibitions were seen only when so called intestino-intestinal inhibitory reflexes were induced. Since CELANDER's and KOCK's studies were confined to the control of jejunal

motility their conclusions are valid only for this part of the intestinal tract as regional differences in the organization of the autonomic nerve supply may exist

The results of CELANDER and KOCK subsequently confirmed by JOHANSSON and LANGSTON (1964) do not imply that the small intestine lacks entirely inhibitory fibres belonging to the sympathetic nervous system They indicate rather that the functional organization of the sympathetic control of the gastrointestinal tract is in all probability far more complex and differentiated than has hitherto been assumed Thus the sympathetic influence exerted when bulbar and suprabulbar sympathetic centres are activated seems to differ considerably from those reflexes originating in the gastrointestinal tract itself It has been well known for several decades that so called intestino intestinal reflex inhibitions can be induced by distension of intestinal loops (cf YOUMANS 1944) It appears that similar reflexes can be induced from most abdominal organs and mesenteries as well as from the peritoneum and possibly even from some skin areas (e g KING 1924 DOUGLAS and MANN 1941) CHANG and HSU (1942) suggested that such reflexes were mediated via the spinal medulla and the results of FREUND and SHEEHAN (1943) pointed in this direction also KUNTZ and SACCOMANNO (1944) on the other hand claimed that these intestino intestinal reflexes were synaptically relayed in the abdominal ganglia mainly the coeliac and inferior mesenteric ganglia Neurophysiological studies confirm that synaptic connections do exist in prevertebral sympathetic ganglia (e g JOB and LUNDBERG 1952 BROWN and PASCOE 1952) but it does not follow from this that they form the substrate of the intestino intestinal reflexes as suggested by KUNTZ and SACCOMANNO FREUND and SHEEHAN stressed that it is often difficult technically to produce a complete decentralization of the abdominal sympathetic ganglia which raises the possibility that the responses studied by KUNTZ and SACCOMANNO might after all have been dependent upon bulbo spinal connections It was shown recently by JOHANSSON and LANGSTON (1964) that spinal anaesthesia completely abolishes the intestino intestinal reflexes in cats while they are unaffected or even enhanced by transsections of all connections between bulbar and spinal parts of the medulla These findings confirm the view put forward in 1942 by CHANG and HSU that all these intestinal inhibitory reflexes elicited from the abdominal organs are mediated via the spinal medulla presumably forming segmental reflex arcs Quite recently JOHANSSON JONSSON and LJUNG (1965) demonstrated that these spinal intestino intestinal reflex arcs could be blocked by topical stimulations in restricted parts of the medulla oblongata indicating that these spinal reflex connections are exposed to descending inhibitory fibres

It has also been suggested that the *vagal nerves*, often considered to be a purely excitatory link in the extrinsic control, carry inhibitory fibres to the small intestine. Thus BAYLISS and STARLING (1899) suggested that the vagus may contain two sets of efferent fibres to the intestine, one inhibitory in function with a short latency and the other excitatory with a long latent period. A similar opinion was put forward by THOMAS and KUNTZ (1926), who studied the effect of vagal stimulations performed in the thorax, and by ALVAREZ, HOSOI, OVERHOLARD and ASCANIO (1929) who stimulated the vagal nerves in the neck in rabbits. In experiments on dogs with intact adrenals and splanchnic nerves, HSU (1944) stimulated the cervical vagus and the intracranial vagal roots and obtained either a few augmented contractions followed by inhibitions or pure inhibitory responses. He concluded that the intestinal inhibitions were due to an activation of inhibitory fibres arising in the medulla oblongata. CRIDER (1946, 1947) observed that with subcutaneous injections of apomorphine in dogs with a Thiry-Vella jejunal loop, the jejunal motor activity was reflexly inhibited simultaneously with the appearence of nausea and vomiting. The inhibitory reflex was unchanged after cutting the splanchnic nerves and could be shown to be vagally mediated. However, it was not settled whether these inhibitory responses could be ascribed to a transient drop in the prevailing activity of vagal excitatory fibres or if they were a consequence of the activation of specific inhibitory fibres. More recently VAN HARN (1963) investigated the effect of vagal stimulation in the neck on the jejunal motor activity in cats in whom the splanchnic nerves and the adrenal glands were intact. He obtained mostly excitatory effects, but occasionally inhibitory responses were seen when the intestinal activity initially was pronounced. Atropine blocked the excitatory responses induced by vagal stimulation and reversed these responses to inhibitions in some experiments. Although the vagally induced inhibitory responses were not seen regularly and appeared after a rather long latency, the author concluded that they were due to an activation of adrenergic inhibitory fibres in the vagal nerves. HARPER, KIDD and SCRATCHERD (1959), on the other hand, could not induce inhibitory responses of a duodenojejunal loop by intrathoracic vagal stimulation in cats whether the splanchnic nerves were intact or not. In addition STAVNEY, KATO, GRIFFITH, NYHUS and HARKINS (1963) did not find any inhibitory responses in the small intestine following intrathoracic vagal stimulation in experiments on dogs in whom the splanchnic nerves were sectioned. The recording technique, however, did not allow registration of tonic intestinal inhibitory responses with any degree of certainty, since intestinal motor activity was recorded as pressure changes with open tipped catheters.

In the above [illegible] it should be noted that the inhibi[illegible]

tory responses sometimes observed upon vagal stimulation were stated to be delayed in onset in many cases. This fact alone suggests an indirect mechanism since the intestinal smooth muscles generally respond promptly to nervous influences, even inhibitory ones, as is the case with the intestino-intestinal inhibitory reflex. Furthermore, the adrenal glands had been left intact in most of these experiments. The inhibitory responses might therefore simply have been caused by reflexely released catecholamines from the adrenals as a consequence of the blood pressure fall induced when the vagal nerves were stimulated in the neck. Whether the vagal nerves really carry any specific inhibitory fibres to the small intestine cannot be settled on the basis of these experiments.

The well established excitatory responses of the small intestine have been obtained by vagal stimulations both at the cervical level (BAYLISS and STARLING 1899, 1901; ALVAREZ *et al.* 1929; HUKUHARA 1931; HSU 1944; GRAY, HENDERSHOT, WHITROCK and SEEVERS 1955; VAN HARN 1963) and at the intrathoracic level below the heart (KLEE 1912; KURÉ *et al.* 1931; BARRY 1933; HARPER *et al.* 1959; STAVNEY *et al.* 1963) in dogs, cats and rabbits. Equally strong motor responses are obtained whether the left or right vagal nerve is stimulated at the neck level, while the motor fibres seem to run mainly in the coeliac branch of the right (posterior) vagus at the subdiaphragmatic level (HARPER *et al.* 1959; STAVNEY *et al.* 1963). The great majority of earlier investigators have studied only the effect of vagal stimulation on the upper part of the small intestine and only a few studies have dealt with the vagal control of the whole small intestine. BAYLISS and STARLING (1901), ALVAREZ *et al.* (1929) and STAVNEY *et al.* (1963) in cats, rabbits and dogs, respectively, found that vagal stimulation induced motor responses in both the proximal and distal parts of the small intestine. In these experiments the sympathetic innervation to the small intestine had been cut, and BAYLISS and STARLING found it difficult, and in most cases impossible, to induce motor responses if the splanchnic nerves had not been previously sectioned. KLEE (1912) and KURÉ *et al.* (1931), on the other hand, stimulated the vagal nerves in dogs and cats, respectively, with intact splanchnic nerves and concluded that the vagus innervates mainly the upper parts of the small intestine. KURÉ *et al.* concluded that the parasympathetic vagal fibres innervate the entire small intestine in some animals but in others the upper parts only.

Thus, opinions diverge as to the parasympathetic vagal innervation of the upper and lower parts of the small intestine. It should be noted, however, that in the above mentioned studies the splanchnic nerves were cut in some but left intact in others. The adrenal glands have also usually been left intact and as catecholamines reflexly released into the blood stream seem to be

an important factor in inducing intestinal inhibition such a mechanism may well have interfered with the intestinal responses obtained by e.g. cervical vagal stimulations. It should also be recalled that the prevailing motility level as well as the stimulation strength and frequency have been said to influence the nature of the intestinal responses both to vagal and splanchnic nerve stimulation (e.g. CONNEL 1961). Thus CULANDER (1959) and KOCK (1959) as mentioned previously stressed the importance of using stimulation frequencies within the physiological discharge rate (i.e. below 8—10 impulses per second) if one is to judge correctly the physiological responses of the intestine to splanchnic nerve stimulation.

The excitatory influence of the vagal nerves has also been studied by observing the effects of vagotomy on the intestinal motility. The results obtained from such experiments are, however, contradictory and studies of e.g. the passage of contrast medium through the small intestine has been found by some investigators to be normal and by others to be retarded (for ref. see POTH and BEAMS 1959, DERBLOM and NYLANDER 1963). Nobody seems to have studied the effect of vagotomy on the motility of different parts of the small intestine.

Although earlier investigations have clearly revealed the existence of excitatory vagal fibres engaged in the extrinsic control of duodenal and jejunal motility, there are thus far no clear cut data concerning the vagal control of ileal motor activity. Thus, generalized conclusions as to extrinsic control of the intestine have often been derived from experiments performed on the upper part only. This fact is all the more remarkable since significant differences have been reported between upper and lower parts of the small intestine. For instance, a decreasing gradient of rhythmicity along the intestine has been reported. Similarly, differences in density of nerve cells in the myenteric plexus, in the content of histamine, choline acetylase, 5-HT and substance P are said to exist (for ref. see HASSELBACH and THOMAS 1961, DANIEL and CHAPMAN 1963).

It is obvious from what has been outlined above that the functional organization of the extrinsic nervous control of the small intestine motor activity far from being a settled question constitutes rather a fairly confused field where carefully controlled quantitative studies are to a great extent lacking. Many of the disagreements can probably be explained by differences in the experimental conditions so that secondarily engaged mechanisms may have been responsible for reactions ascribed primarily to the vagal nerves.

In consideration of the above mentioned observations the purpose of the present investigation was to study the following:

1 and 2. To investigate the vagal control of the small intestinal motor activity with special regard to the extent of the excitatory influence on the upper and lower parts of the small bowel and to establish the reason for a possible difference in the extent of the vagal excitatory fibres to the small bowel.

3. To examine whether the vagal nerves at the neck level contain any inhibitory fibres to the small intestine.

4. To evaluate the probable physiological range of impulse discharge of the fibres distributed to the small intestine.

5. To investigate whether the sympathetic fibres said to join the vagal nerves at the intrathoracic and subdiaphragmatic levels are of any significance for the control of the motility and blood flow of the small intestine.

6. To examine whether the vagal nerves convey any specific vasodilator fibres to the vessels of the small intestine.

CHAPTER II

Methods

a. *Material and anaesthesia*

Experiments were performed on 137 cats of both sexes with body weights varying between 1.7 and 4.8 kg (mean 2.8 kg).

During an initial ether anaesthesia a catheter was inserted into the right femoral vein and the animals were then anaesthetized with chloralose (50 mg/kg) and urethane (100 mg/kg) administered slowly intravenously. The body temperature was kept at 37° to 38° C by means of a thermostatically regulated heating pad placed under the animal and an infrared lamp above the cat. Heparin was given intravenously in doses of 2 to 5 mg/kg body weight. Incidental blood losses were compensated with a Dextran-Tyrode solution.

not to damage the vascular and nervous supply in the mesenteric pedicle of the preserved loops The weight of the intestinal loops when the intraluminal content had been removed varied between 6 and 14 gm The total weight of the small intestine from flexura duodeno jejunalis to the ileocal valve was determined in thirty cats and was found to be 71±4 gm

The contents in the intestinal segments were removed and the distal end of each loop was connected to a glass tube which by way of a rubber tube and an adaptable pressure reservoir led to a piston recorder The systems were filled with Tyrode solution The pressure within the intestinal segments could by raising or lowering the reservoirs be set at any desired level By keeping the reservoirs at about 10 to 15 cm of water a good spontaneous motor activity was usually present The reservoirs had a comparatively large cross sectional area so that the pressure within the loops was kept at a rather constant level even when their volume changed markedly However in these experiments in which quantitative measurements of the volume changes were performed graded reservoirs with lesser cross sectional area were used to allow a more exact estimation of the volume changes obtained Here the quantitative estimations of the intestinal motor responses were done by measuring the change in the height of the water column in the graded reservoirs Thus the intestinal motor activity discussed in the present study from both quantitative and qualitative point of view was recorded by measuring the volume changes in the respective part of the small intestine The gastric motility was recorded by means of latex balloon introduced into the stomach via the oesophagus The balloon inflated with 30—40 ml of air was connected to a water manometer recording on the kymograph

Catechol amines from the adrenal glands are known to be potent factors in producing inhibition of intestinal motility Therefore it was found desirable in some of the present experiments to eliminate the secretion from these glands This was done in most experiments by ligating the vessels of both adrenal glands In other experiments the vessels of the right adrenal gland were ligated and the nerves to the left adrenal cut In this way a reflex discharge of catechol amines was prevented without disturbing the secretion of the corticoid hormones from the left adrenal gland

In some experiments it was desirable to perform a complete block of the sympathetic outflow to the small intestine In addition to administration of guanethidine and dihydroergotamine (see below) this was performed by means of spinal anaesthesia This was accomplished by subdural injection of 1 to 1 5 ml of a 2% solution of tetracain *via* a thin polyethylene catheter inserted in the subdural space through a lumbar laminectomy with the tip at the lower thoracic level

c *Recording of blood pressure and intestinal blood flow*

The mean arterial blood pressure was recorded continuously by means of a mercury manometer connected to one of the femoral arteries

The intestinal blood flow was determined by measuring the total venous outflow from the jejunal and ileal loops The superior mesenteric vein was dissected free up to the entrance of the splenic vein into the portal vein and all its branches except those from the intestinal segments to be studied were ligated A wide bore polyethylene tube was inserted in the distal end of the superior mesenteric vein and the total venous outflow was diverted to a closed perspex drop chamber in which the blood dropped through silicone oil (LUNDGREN 1958) The venous outflow was returned to the animal *via* one of the external jugular veins or in some cases *via* the left femoral vein An optical drop counter applied to the drop chamber operated an ordinate writer which registered the drop rate on a kymograph (CLEMENTZ and RYBERG 1949) The heights of the ordinates are *inversely* proportional to the rate of the blood flow The height of the ordinate was converted directly to flow in ml min by means of a calibration curve During many experiments the venous outflow was checked repeatedly with a graduated cylinder and a stop watch The flow resistance of this system was minimized by using siliconized rubber tubes as wide and as short as possible The operative procedure for inserting the blood flow recorder required less than one minute

Peripheral resistance units (PRU) were calculated as the ratio of mean arterial blood pressure (mm Hg) to blood flow (ml min/100 g tissue) neglecting venous pressure

tissues The plastic tube was filled with paraffin oil at body temperature and the thoracotomy covered with a plastic sheet

The *subdiaphragmatic* portions of the vagal nerves were dissected free from adjacent tissues immediately below the diaphragm and cut as far proximally as possible The distal ends of the nerves were passed through an electrode as described above For technical reasons only one nerve at a time was stimulated usually the right (posterior) nerve since in most experiments it proved impossible to initiate any intestinal motor activity by left vagal stimulation During the preparation great care was taken to protect the nerves from drying cooling and from mechanical damage

Splanchnic nerve stimulation was usually performed by stimulating the distal ends of the greater and the lesser splanchnic nerve on the left side as it is often difficult technically to prepare the splanchnic nerves on the right side without traumatizing the animals too much However in a few experiments the splanchnic nerves were dissected free on both sides and centrally cut The right splanchnic nerves were then brought under the inferior caval vein over to the left side and all the splanchnic nerves were placed on the same electrode without traction or damage In order to stimulate the splanchnic nerves with the abdomen closed and to avoid accidental stimulation of adjacent tissues the following arrangement was performed A plastic tube containing a coil formed silver electrode was introduced into the abdominal cavity *via* a small separate opening in the left side of the abdominal wall The peripheral ends of the nerves were cautiously drawn up into the tube and placed on a bipolar silver electrode after which the tube was filled with paraffin oil at body temperature

It was considered to be of great interest to compare the effects on intestinal motility and blood flow with the so called intestino intestinal inhibitory reflex i e the inhibition of tone and rhythmicity produced in one isolated intestinal segment when some other isolated segment is distended This inhibitory reflex is easily reproduced by direct stimulation in the afferent direction of the periarterial nerve fibres distributed to the distended intestinal loop (Kock 1959) Therefore in most experiments a small mesenteric pedicle was left intact between the jejunal and ileal loops Intestino intestinal inhibitory reflexes could then be induced repeatedly by a graded afferent stimulation of the mesenteric pedicle

The nerve trunks usually remained fully excitable for several hours The electrical stimulations were all performed with bipolar silver electrodes connected to a Grass Stimulator (Type S4) delivering square wave stimuli with variable impulse frequency duration and intensity Care was taken to assure

rhythmic activity decreased somewhat after vagal section. A similar though less pronounced decrease of ileal motor activity was also noticed in 5 animals. Furthermore, fairly soon after this decrease in jejunal or ileal motor activity it gradually returned again in most cases usually to the preceding levels so that in the long run no distinct differences could be observed between intestinal activity before and after vagal section.

II. Effects of vagal stimulation in the neck

a) *Effects of variation in pulse-duration and intensity*

In 59 experiments the effects of efferent vagal stimulation in the neck on jejunal and ileal motor activity were investigated. The animals were prepared as described in Chapter II, with intact splanchnic nerves and adrenal glands. In 13 of these experiments the gastric motor activity was recorded as well. The distal ends of the two vagal nerves were stimulated intermittently in the neck with square wave pulses at a constant frequency, usually 4 or 8 impulses per second, while varying systematically in a step-wise fashion voltage between 1 to 10 volts, sometimes 20, and pulse duration between 0.1 and 10.0 msec. In practically all of these experiments vagal stimulations induced an immediate and generally strong motor activity in the jejunum. In the ileum, on the other hand, motor responses were induced in only 50 per cent of the experiments. Thus, no significant responses to vagal stimulation occurred in half of the animals. Further, when present, the ileal motor responses to vagal stimulation were often not as distinct in intensity as those in the jejunum. In the stomach vagal stimulations produced distinct motor responses in all the 13 animals in whom gastric motility was recorded. Fig. 1 shows an experiment in which the responses to vagal stimulations were recorded simultaneously in the stomach, the jejunum and the ileum. In this particular experiment the ileal responses were relatively pronounced while in the experiment shown in Fig. 3 the ileal motor responses were negligible, at least at lower rates of stimulation. No significant difference in response in the respective intestinal sections or the stomach was seen if the vagal nerves were stimulated separately and the effects compared.

The strength-duration relationship for the vagal parasympathetic fibres to the smooth muscles of the stomach was studied in 13 animals and for the jejunum and the ileum in altogether 41 animals. The stimulation frequency was kept constant at 4 imp/sec as this frequency produced fairly marked and reproducible motor effects. The voltage and pulse duration was varied in a regular fashion and the pulse duration required for a given voltage to induce barely visible motor responses in the respective parts of the gastro-

tissues The plastic tube was filled with paraffin oil at body temperature and the thoracotomy covered with a plastic sheet

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The nerve trunks usually remained fully excitable for several hours The electrical stimulations were all performed with bipolar silver electrodes connected to a Grass Stimulator (Type S4) delivering square wave stimuli with variable impulse frequency duration and intensity Care was taken to assure

that the distal electrode contact was always used as the cathode whenever stimulations of efferent fibres were performed

c *Administration of drugs*

Acetylcholine (Roche) and l-adrenaline hydrochloride were infused into the inferior caval vein by means of a fine catheter introduced into one of the femoral veins. Constant infusions were given with a motor driven syringe at various rates. All dosage values of l-adrenaline are given below as the HCl salt. In some experiments acetylcholine was also infused intraarterially. For this a plastic catheter was introduced into the right renal artery with the tip at the origin of superior mesenteric artery. The inferior mesenteric artery had previously been ligated in these experiments so that the jejunal and ileal loops of the small intestine received their blood supply via the superior mesenteric artery only.

The various solutions were prepared immediately before the start of each experiment.

Solutions of guanethidine (Ismelin Ciba) and dihydroergotamine (Sandoz) were injected into the inferior caval vein.

The preparations described above usually lasted from one and a half to three hours. Great care was taken to avoid cooling and drying of the organs. When the operative procedures were finished the abdominal wall was closed with clips. The intestinal loops were left *in situ* inside the abdominal cavity. The experiments were not started until the intestinal motor activity and blood flow had stabilized at a fairly constant basal level.

CHAPTER III

Effects of cervical vagal nerve section and stimulation on the jejunal and ileal motility

To compare the vagal control of motor activity of the jejunum and the ileum the following experiments were performed The effect of section of the vagus in the neck was first studied while recording separately the motor activity of jejunal and ileal segments (A) The effect of graded stimulations of the distal ends of the vagal nerves in the neck on jejunal and ileal motor activity was then investigated (B) Here special attention was paid to the effect of vagal stimulation on intestinal motor activity when voltage and pulse duration were varied before and after atropine and in animals with and without adrenal glands (B a) The effect of changes in stimulation frequency alone upon intestinal motility was also studied (B b) On the basis of these latter findings attempts were made to evaluate the physiological range of impulse discharge in the vagal fibres to the intestinal smooth muscles as judged from the characteristics of the frequency response curve obtained

A Effects of sectioning the vagal nerves in the neck

The spontaneous motor activity of the jejunum and the ileum usually differed initially under the prevailing experimental conditions The jejunum showed as a rule an intense rhythmical activity at a fairly constant basal tone level The motor activity of the ileum was usually less pronounced while the basal tone sometimes varied considerably during the course of the individual experiment The extent of the basal tone level of the two intestinal segments here was estimated approximately by recording the extent of intestinal relaxation produced by a supramaximal dose of adrenaline intravenously

In order to find out if the vagal nerves exert any tonic influence upon the small intestine of the anesthetized animals the nerves were sectioned in the neck while the intestinal motor activity was recorded continuously in a total of 41 experiments In most of these experiments there was no major change in intestinal motility or basal tone after vagal section In 5 animals however in whom the jejunal activity was especially pronounced its tone and

rhythmic activity decreased somewhat after vagal section A similar though less pronounced decrease of ileal motor activity was also noticed in 5 animals Furthermore fairly soon after this decrease in jejunal or ileal motor activity it gradually returned again in most cases usually to the preceding level so that in the long run no distinct differences could be observed between intestinal activity before and after vagal section

II Effects of vagal stimulation in the neck

a) *Effects of variation in pulse-duration and intensity*

In 50 experiments the effects of efferent vagal stimulation in the neck on jejunal and ileal motor activity were investigated The animals were prepared as described in Chapter II with intact splanchnic nerves and adrenal glands In 13 of these experiments the gastric motor activity was recorded as well The distal ends of the two vagal nerves were stimulated intermittently in the neck with square wave pulses at a constant frequency usually 4 or 8 impulses per second while varying systematically in a step wise fashion voltage between 1 to 10 volts sometimes 20 and pulse duration between 0 1 and 10 0 msec In practically all of these experiments vagal stimulations induced an immediate and generally strong motor activity in the jejunum In the ileum on the other hand motor responses were induced in only 50 per cent of the experiments Thus no significant responses to vagal stimulation occurred in half of the animals Further when present the ileal motor responses to vagal stimulation were often not as distinct in intensity as those in the jejunum In the stomach vagal stimulations produced distinct motor response in all the 13 animals in whom gastric motility was recorded Fig 1 shows an experiment in which the responses to vagal stimulations were recorded simultaneously in the stomach the jejunum and the ileum In this particular experiment the ileal responses were relatively pronounced while in the experiment shown in Fig 3 the ileal motor responses were negligible at least at lower rates of stimulation No significant difference in response in the respective intestinal sections or the stomach was seen if the vagal nerves were stimulated separately and the effects compared

The strength duration relationship for the vagal parasympathetic fibres to the smooth muscles of the stomach was studied in 13 animals and for the jejunum and the ileum in altogether 41 animals The stimulation frequency was kept constant at 4 imp /sec as this frequency produced fairly marked and reproducible motor effects The voltage and pulse duration was varied in a regular fashion and the pulse duration required for a given voltage to induce barely visible motor responses in the respective parts of the gastro

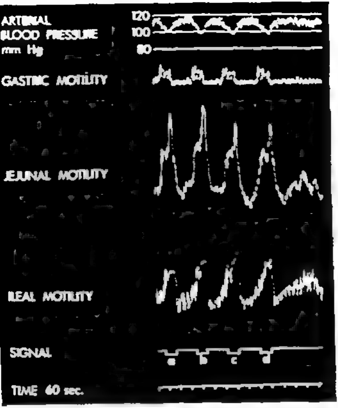

Fig 1 Cat 1 9 kg Choralose urethane Effect of efferent stimulation of the left vagal nerve in the neck with 4 imp /sec 8 V and 2 0 4 0 10 0 and 1 0 msec (a b c and d respectively) on gastric jejunal and ileal motility

Both vagal nerves were cut in the neck The adrenal glands and the splanchnic nerves were intact

intestinal tract was determined In this way threshold stimuli could be evaluated approximately The threshold intensity required to produce excitatory responses in the stomach could be estimated in all the 13 animals and for the jejunum in 36 out of the 41 animals As the ileum in half of the animals did not respond with an increased activity to stimulation even when all efferent vagal fibres were excited and exhibited only weak responses in others such experiments could not be utilized for determining stimulation thresholds However in 10 animals in whom supramaximal vagal stimulation at 4 impulses per second elicited ileal motor responses about as pronounced in extent and onset as those in the jejunum it was possible to evaluate the stimulation strength needed to produce barely visible motor responses to vagal stimulation in the ileum

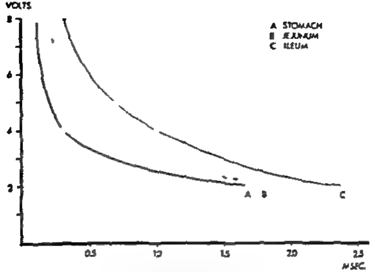

Fig 2 Strength-duration curves for the mean threshold values of excitatory effects of cervical vagal stimulation on gastric jejunal and ileal motility
For number of experiments see text

The strength duration relationship for the vagal motor fibres to the stomach the jejunum and the ileum are illustrated in Fig 2 the curves representing the mean values obtained from all the experiments which were suitable for such estimations As seen in Fig 2 the strength duration relationship in the motor fibres to the stomach the jejunum and the ileum were not exactly the same but there is some overlap between the curves when the spread around the mean values for all the experiments is taken into account It was on the other hand often found that in the individual experiment the voltage needed to produce a gastric response was lower than that needed to produce an ileal motor response making it likely that the motor fibres running to the stomach have on an average slightly lower thresholds than those running to the ileum

One main reason for this systematic exploration of the effects of varying intensities and pulse durations was to see whether the vagal nerves might contain also *inhibitory* fibres to the intestinal smooth muscles as has been found to be the case with the stomach (e g MARTINSSON and MUREN 1963) However even when changing the stimulation parameters over a wide range including quite high intensities it was impossible to elicit any inhibition of the jejunal motor activity or even a partial supression of the excitatory responses which could be taken as evidence for an activation of high thresholds

inhibitory fibres (compare MARTINSSON and MURRY 1963) In the ileum however inhibitory effects were noted in 10 out of the 59 animals but it should be stressed that they appeared first after a latency of as long as 20 to 25 seconds Furthermore these ileal inhibitions appeared quite irregularly and with no clear correlation to a given voltage — pulse duration as was the case both with the vagal excitatory responses In experiments in which the adrenal glands were excluded from the circulation the inhibitory motor responses disappeared

In order to explore this problem further atropine (0 2 to 0 5 mg/kg) was injected intravenously in 17 of the 59 animals since it is known that such a procedure reveals the vagal inhibitory fibres to the stomach (e g MARTINSSON and MURRY 1963) This was performed in order to block the effect of the cholinergic excitatory fibres to the intestine which could then possibly reveal existing non cholinergic vagal inhibitory fibres exerting a direct inhibitory influence on the intestinal smooth muscles (It should be noted that if hypothetical vagal fibres instead exert their inhibitory action on the local excitatory ganglionic cells atropine will not be able to reveal their existence In that case however they would be different in their organization than the vagal inhibitory fibres distributed to the stomach) Vagal nerve stimulations at different pulse durations and voltages were then repeated In none of these experiments did any inhibitory responses appear in the jejunum and only in one out of 17 animals were there slight inhibitory responses in the ileum which were weak and appeared first after a latency of 20 seconds The fact that adrenaline injection still could produce inhibitory responses showed that atropine *per se* had not caused any complete intestinal relaxation which of course would have made impossible any neurogenic inhibitory responses

It seems highly doubtful if the above mentioned long latency inhibitory responses of the ileum which occurred quite irregularly in some of the present experiments were really due to excitation of specific vagal inhibitor fibres the more so as they were abolished when the adrenal glands were excluded from the circulation

b) *Effects of variation in stimulation frequency*

In another series of 20 experiments one or both of the distal ends of the cut vagal nerves were stimulated in the neck with constant supramaximal voltage and pulse duration to ensure that all the motor fibres to the intestine were activated In these experiments the *frequency* was varied between 1 and 60 imp /sec while recording in most experiments both jejunal and ileal motor activity as well as intestinal blood flow This series was performed in order to explore the frequency response characteristics of the intestinal motor

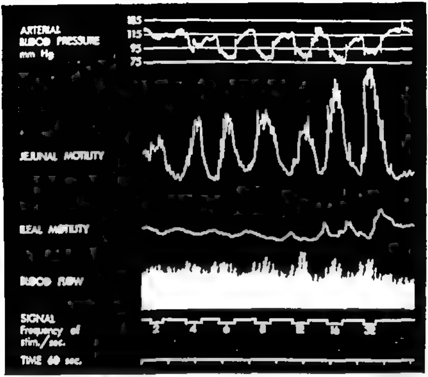

Fig 3 Cat 2 6 kg Chloralose urethane Jejunal and ileal motility and blood flow during maximal efferent cervical vagal stimulations with a stepwise increase in frequency as indicated Impulse duration and intensity kept constant at 3 msec and 10 V respectively The heights of the jejunal motor responses at the two highest frequencies are not directly proportional to the values of the absolute motor responses at these frequencies Note the difference in responses between jejunum and ileum

Both vagal nerves were cut in the neck The adrenal glands and the splanchnic nerves were intact Resting intestinal blood flow 41 ml/min/100 g intestinal tissue

fibres and to see whether the vagal nerve supply might affect the intestinal blood vessels also

Fig 3 shows an experiment in which the jejunal and the ileal activity as well as the blood flow through both these intestinal loops were recorded while the left vagal nerve was stimulated at increasing frequencies Each nerve stimulation was maintained for a period of about 30 to 40 seconds so as to let the intestinal response reach a plateau and at each stimulation period the impulse rate was increased in a stepwise fashion as shown in the figure With this unilateral vagal stimulation the motor responses in the jejunum were enhanced with increasing frequency reaching a maximum at 32 imp /sec in

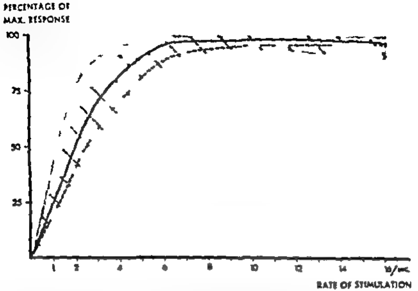

Fig 4 Frequency response curves for the jejunal motor responses obtained by unilateral (dashed line) and bilateral (continuous line) efferent cervical vagal stimulation All effects calculated in percentage of the maximum responses obtained The curves are based on the results obtained in 15 animals Shaded areas indicating all individual variations Note the comparatively small difference between the two curves indicating a rather appreciable convergence of the fibres from the two vagal nerves upon the effector cells in the intestine For discussion see text

this experiment but as much as some sixty per cent of the maximal response was elicited at 4 to 8 imp /sec In this experiment there were no significant ileal responses at least not at the lower frequencies which was a typical finding in about fifty per cent of the experiments as previously mentioned However in experiments in which distinct motor responses were obtained in the ileum these responses increased with increasing stimulation frequencies just as seen in the jejunum The intestinal vascular resistance did not decrease as a result of the vagal stimulations in the neck The influence of the vagal nerves on the blood vessels of the small intestine will be discussed in more detail in Chapter V

The correlation between the stimulation frequency and the jejunal motor response in 15 technically successful experiments in which both (continuous line) or only one (dashed line) of the vagal nerves were stimulated is illustrated in the diagram of Fig 4 in form of the mean values The jejunal motor responses expressed as an approximate percentage of the maximum response are plotted along the ordinate and the stimulation frequencies along the abscissa It is evident from this figure that by far the steepest increase in the

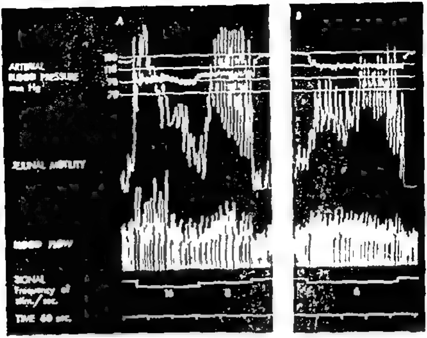

Fig 5 Cat 2 9 kg Chloralose urethane Effect of efferent prolonged vagal neck stimulation (4 V 3 0 msec) at high and low frequencies on jejunal motility and blood flow Note the gradual decrease of the jejunal motility at high frequency stimulation and the increase of motility on shifting to a lower frequency (A) Note also that the increased motor activity at the fairly low frequency stimulation is well maintained during a prolonged period of stimulation (B)

Both vagal nerves were cut in the neck The adrenal glands and the splanchnic nerves were intact "Resting" intestinal blood flow 44 ml min/100 g intestinal tissue

intestinal motility was elicited when the rate was increased up to about 4 to 6 imp /sec and with bilateral vagal stimulation nearly maximal effects were reached on an average at 6 to 8 imp /sec Stimulation with higher frequencies up to 16 to 32 imp /sec resulted only in small additional increases of the motor activity when unilateral vagal stimulations were performed It is obvious here that even slight damage to the nerve fibres or minor interferences with effector sensitivity will tend to depress the effector response to a given nerve stimulation thereby flattening and displacing the frequency response curve to the right It seems reasonable that the frequency response relationship in the intact animals is if anything placed somewhat to the left of that illustrated in Fig 4

The high frequency responses could not be maintained for any length of time but started regularly to decline after about a minute of continued stimulation This is illustrated at *A* in Fig 5 in which the left vagal nerve was

stimulated first at 16 imp./sec. and continued for 150 seconds. The immediate effect of this high frequency stimulation was a strong increase of jejunal motility but after about 40 to 50 seconds this increased motility declined gradually and had almost vanished after some 150 seconds of continued stimulation. Without interruption of stimulation the frequency was shifted to 8 imp./sec. and the jejunal motor activity now increased again to about the level seen initially at 16 imp./sec. At this lower frequency the response was well maintained during the whole stimulation period of about 120 seconds. This shows that the declining intestinal motor response to high rates of stimulation was not due to any damage of the fibres or to any exhaustion of the effector cells as then the response would have declined further at the shift to the lower frequency. — At *B* in Fig. 5 the vagal nerve was stimulated with 6 imp./sec. for about 4 min. At this fairly low frequency the increased motor activity stayed practically the same throughout the whole period of stimulation.

In general, even when frequencies as moderate as 12 imp./sec. were used the motor responses slowly tended to fail as shown for 16 imp./sec. in Fig. 5 A and the higher the frequency the more rapidly this decline appeared. For instance with a frequency of 60 imp./sec. the induced motor activity started to decrease after only 10 to 20 seconds. The motor responses could then be regained during continued stimulation if the impulse rate was reduced to below 10 imp./sec. or if the nerve stimulation was stopped for a while.

In the present experiments intestinal inhibitory responses could not be induced when the vagal nerves were stimulated in the neck with supramaximal intensities at frequencies between 1 and 60 imp./sec.

In summary these experiments have shown that under the prevailing experimental conditions there seem to be no or at least only insignificant tonic vagal activity to the intestine in the majority of the animals. In only one tenth of the cats did vagal section produce a clearcut though transient depression of intestinal activity.

Vagal stimulation in the neck elicited prompt jejunal motor responses in all the cases but distinct ileal responses appeared in only about half of the animals. In the majority of the cases the motor responses were quantitatively greater in the jejunum than in the ileum. In a few of the experiments vagal stimulation sometimes induced ileal inhibitions but these effects were delayed and irregular. On the whole no evidence of inhibitory vagal fibres to the small intestine could be obtained despite the fact that the stimulus strength and frequency was varied over a wide range.

The strength-duration relationship for the vagal excitatory fibres to the jejunum and ileum was practically the same but the thresholds tended to be slightly higher than those of the gastric excitatory fibres.

Virtually maximal motor responses to bilateral vagal stimulation were elicited at frequencies as low as some 6 to [illegible] imp/sec with the maximum around 10 to 16 imp/sec. Thus the steepest part of the frequency response curve fell between 0 and [illegible] imp/sec. At rates below some 10 imp/sec the motor responses were well maintained over even prolonged periods of stimulation. Above 12 to 16 imp/sec the induced motor responses gradually declined after some 20 to 60 seconds of stimulation. Shifting to a frequency below 10 imp/sec caused the responses to appear again.

C. Comments

From histological studies of the vagi it is known that the great majority of the fibres in the abdominal branches are unmyelinated and less than 10 per cent of the fibres are efferents. There is only a small number of myelinated fibres but the majority of these are afferents with a diameter below 6 μ (AGOSTINI, CHINNOCK, DE BURGH DALY and MURRAY 1957; HOFFMAN and SCHNITZLEIN 1961). The voltage-pulse duration values needed to activate the efferent vagal motor fibres to the gastro-intestinal tract are slightly lower than those needed to excitate somatic afferent pressor fibres (non-medullated C-fibres) and postganglionic sympathetic fibres but distinctly higher than the somatic depressor fibres (group III fibres) (cf. JOHANSSON 1962). The threshold stimuli required to elicit motor responses in the stomach, jejunum and ileum did not differ significantly. Therefore the vagal efferent fibres to the smooth muscle of the gastrointestinal tract seem to be of largely the same type with respect to threshold and diameters, possibly, though the fibres to more oral sections have slightly lower thresholds, if anything. In any case, whatever differences exist they are not distinct enough as to allow for clearly selective activations of e.g. only the excitatory fibres to the stomach.

The smooth muscles of the small intestine, as well as all other autonomic effectors in the cat, respond promptly to nerve stimulations with a latency of only a few seconds. This is also true for the intestinal inhibitory responses, e.g. the neurogenic intestinal inhibition caused by activation of the so called intestino-intestinal inhibitory reflex. It is therefore most unlikely that the ileal inhibitions with a latency of 20 to 25 seconds seen in a few experiments were due to excitation of vagal inhibitory fibres. From earlier experiments it is known that the intestinal smooth muscles are inhibited by even small amounts of adrenaline (KOCK 1959). YOUMANS, AUMANN, HANEY and WYNIA (1939) studied the effect of intravenously infused acetylcholine on the motor activity of the small intestine, and obtained an intestinal inhibition after a transient excitatory response. The inhibitory response was explained as an

effect of reflexly released adrenaline caused by the concomitant blood pressure fall. Similarly vagal stimulation in the neck in the present experiments caused a lowering of the blood pressure due to the inhibition of the heart activity, and this blood pressure fall will produce a reflex activation of the vasomotor center by way of the baroreceptors. It is therefore most likely that the delayed ileal inhibitions were due to a reflex release of catechol amines from the adrenal medulla as a consequence of the blood pressure fall. Such a view is strongly supported by the fact that the inhibitions disappeared when the adrenal glands were excluded, after which vagal stimulation produced only excitatory responses. It could be argued that the jejunal motor activity should then also be inhibited by the released catecholamines. The complex relationship between the motor activity in jejunum and ileum will be discussed in detail in the next chapter.

It might also be suggested that the decreased intestinal blood flow as a result of the vagal effect on the heart and the lowering of the blood pressure might depress the intestinal motor activity. However according to KOCK (1959) the intestinal blood flow usually has to be reduced to some twenty five per cent or less of the "resting" blood flow before this factor alone induces a distinct intestinal inhibition. The reduction of the intestinal blood supply as a result of the blood pressure fall in these experiments was too small to be able to induce any ileal inhibitions.

The *physiological discharge rate* of the autonomic nervous system has been studied by several investigators (for ref. see FOLKOW 1957, VON EULER 1959 and HILLARP 1960). Most earlier studies have, however, dealt with the sympathetic nervous system in which resting tonic discharge seems to be of the order 1 to 2 imp./sec. with maximum discharge rates around some 6 to 10 imp./sec. There are only few studies dealing with the discharge rate in the parasympathetic system. GARRY and GILLESPIE (1955) investigated the parasympathetic influence on the rabbit's colon *in vitro*. They obtained maximal motor responses by stimulating the pelvic nerves at 10 imp./sec. This frequency for the maximal response corresponds well with that found in the present experiments when bilateral vagal stimulations were performed. In most of the present experiments eighty to ninety per cent of the maximal response was obtained on bilateral stimulation at rates as low as 6 to 8 imp./sec. (Fig. 4). Thus small variations of the impulse rates in the low frequency range elicited considerable changes of the intestinal motor activity. On the other hand stimulation rates exceeding 12 imp./sec. added as a rule very little to the motor responses obtained at 10 imp./sec. Moreover, it was generally observed that the intestinal motor activity declined and successively approached the preexcitation level of tone when the vagal nerves were sti-

mulated at such high impulse rates for more than a half to one minute If however the vagal fibres were stimulated continuously at rates below 10 to 12 imp /sec the effector responses were well maintained for a considerable length of time

The failing intestinal motor response at high discharge rates can hardly be ascribed to any exhaustion of the smooth muscle effectors as the intestinal motor activity could be kept at a constant high level when prolonged vagal stimulation at rates below some 12 imp /sec was performed It might be suggested that the smooth muscles became exhausted only if they were forced to a definitely maximal response If so intestinal tone would be expected to decrease either to the no doubt easily maintained level obtained at impulse rates of say 6 to 8 imp /sec or decrease temporarily to a still lower level only to again increase after a period of rest However on prolonged stimulations at high rates the effector response gradually vanished completely or almost completely and did not increase again until the frequency during continued stimulation was shifted to values below 10 to 12 per second at which time maximal or nearly maximal responses could be induced again and maintained. Alternatively if the high rate of stimulation was interrupted for only a brief period motor responses could again be induced It thus appears that it is not the effector cells which tend to fail when they are forced to very high rates of discharge True damage of the stimulated fibres can be excluded as then the motor responses would decline still more when the rate of stimulation was decreased Further since it is known that impulses of still higher frequencies can be transmitted along autonomic fibres without failure it seems likely that the failing motor responses to high frequency vagal stimulation depends upon an inability of the parasympathetic synapses and/or the postganglionic neuroeffector junctions to liberate a constant quantity of the cholinergic transmittor per stimulus when driven at rates beyond the physiological rates (see also FOLKOW 1952)

From the facts discussed above it can be concluded that the physiological discharge range of the vagal fibres to the small intestine is probably of the same order of magnitude as that previously found for the sympathetic nervous system i e from zero up to some 8 to 10 imp /sec (FOLKOW 1952) It is of course possible that phasically still higher frequencies are induced for brief periods in order to produce a rapid onset of effector activation as seems to be the case with the sympathetic nervous system (IGGO and VOGT 1960) If this is the case the results of BLAIR HARPER KIDD and SCRATCHERED (1959) are of considerable interest They state that intestinal motor responses elicited with vagal stimulation at low rates (1 to 5 imp /sec) were potentiated by a brief preceeding period of high stimulation rate (20 to 50 imp /sec) and

this potentiation was found to last up to some 10 minutes. If such a facilitating mechanism exists in the intact organism phasically induced high rates of discharge at the onset of e.g. a reflex excitation might to some extent influence the subsequent response to a low frequency tonic discharge.

From the present experiments it is difficult to estimate the resting discharge in the vagal fibres to the smooth muscle cells under physiological conditions. However, the fact that vagal section did induce only a moderate or slight decrease of the intestinal activity in a few of the present experiments and no influence at all in most of the experiments indicates a low rate of tonic vagal discharge. This seems to be the fact under the prevailing experimental conditions but it seems likely that it is also true in the intact animal where an increased activity in the vagal nerves would be appropriate only with food intake and digestion.

CHAPTER IV

Comparison between the extrinsic nervous control of the jejunal and ileal motility

In the previous chapter it was shown that vagal stimulation in the neck elicited motor responses in the jejunum in essentially all experiments but only in a half of them in the ileum. Further, in the animals in whom motor responses were obtained in the ileum these were usually quantitatively less extensive than those in the jejunum for any given stimulation rate.

The differences between the results obtained in jejunum and ileum can be due to several conceivable factors. Thus, the sensitivity of the effectors to the cholinergic vagal transmittor acetylcholine may be different in the jejunum and the ileum. Further, it is possible that the ileum is selectively, or at least to a higher degree than the jejunum, exposed to an inhibitory influence, humoral or nervous, which can counteract the excitatory influence elicited by vagal stimulation. It is known, for instance, that the jejunum is inhibited by quite small amounts of adrenaline reflexly released from the adrenal glands (cf. KOCK 1959). If the ileal smooth muscles are still more sensitive to circulating adrenaline than those of jejunum, such a factor might explain the differences in response obtained by vagal excitation. It is also possible that inhibitory splanchnic nerve fibres are distributed preferentially to the ileum and that the activity of these inhibitory fibres can effectively counteract the excitatory vagal effect. If this is so, one would have to assume a difference in the sympathetic innervation of upper and lower parts of the small intestine, as direct stimulations of the splanchnic nerves at physiological frequencies do not induce any significant and immediate inhibition of the vagotomized jejunum but only a clearcut and well graded vasoconstrictor response (KOCK 1959, CELANDER 1959, JOHANSSON and LANGSTON 1964). Finally, the supply of vagal fibres to different intestinal sections might vary in different animals so that the whole small intestine is vagally innervated in some animals and essentially only the upper portions in others, as suggested by KURÉ *et al.* (1931).

The experiments described in this chapter were carried out to analyse which of the above mentioned factors is responsible for the differences obtained between the jejunal and ileal motor activity by vagal stimulation in the neck.

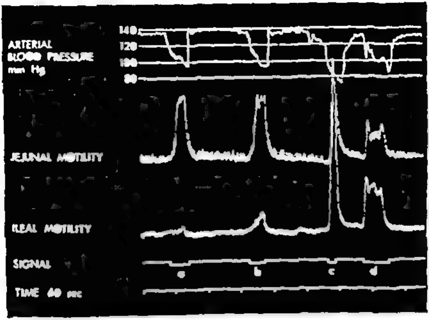

Fig 6 Cat 2 2 kg Chloralose urethane Effects of efferent cervical vagal stimulations (signals a and b) with 10 imp /sec 10 V and 2 0 msec and of intraarterial injection and infusion of [illegible] γ of acetylcholine (signals c and d respectively) on jejunal and ileal motor activity Note that vagal stimulation induced intense motor responses in the jejunum but hardly noticeable responses in the ileum and that acetylcholine induced motor responses in jejunum and ileum of almost the same magnitude

Both vagal nerves were cut in the neck The adrenal glands were ligated bilaterally The splanchnic nerves were intact

A Effects of acetylcholine infusion on jejunal and ileal motility

The excitatory effect of acetylcholine on the smooth muscles of the jejunum and ileum was compared in 17 experiments The drug was injected or infused either intravenously or intraarterially The adrenal glands were excluded to avoid reflex release of adrenaline as a consequence of the acetylcholine induced hypotension The vagal nerves were stimulated in six animals and the motor responses obtained in the jejunum and ileum were compared with those induced by acetylcholine The amounts of acetylcholine injected varied between 0 5—5 γ/kg of body weight but in some animals supramaximal doses up to 100 γ/kg were also injected

Fig 6 shows a typical experiment in which the responses elicited by vagal stimulation in the neck were compared with the responses induced by intra arterial injections of acetylcholine in an adrenalectomized cat with the splanchnic nerves left intact At *a* and *b* the left vagal nerve was stimulated with 10 V [illegible] msec and 10 imp /sec It can be seen that these stimula

tions elicited intense motor responses in the jejunum but a hardly noticeable response of the ileum at a and a small response at b. At c 5 γ/kg of acetylcholine was injected and at d a total of 10 γ was infused slowly intra arterially. As seen from the figure the motor responses elicited by these doses of acetylcholine appeared simultaneously and were of approximately the same magnitude in the jejunum and the ileum if anything they were bigger in the ileum. This is in striking contrast to the results of vagal stimulation.

In 16 out of the 17 experiments threshold motor responses appeared in the jejunum and the ileum with the same doses of acetylcholine and were of the same magnitude at respective doses when the acetylcholine concentrations were increased. The sensitivity of the smooth muscles of the jejunum and the ileum to acetylcholine thus appeared to be the same throughout also in those experiments in which vagal stimulation in the neck induced comparatively smaller responses in the ileum than in the jejunum or no responses at all.

B Effects of adrenaline infusion on jejunal and ileal motility

These experiments were performed on 14 acutely vagotomized cats the purpose being to compare the sensitivity of the jejunum and ileum to adrenaline. The adrenal glands were excluded from the circulation by ligatures but the splanchnic nerves were initially left intact. The blood pressure jejunal and ileal motility and intestinal blood flow were recorded continuously. Adrenaline was infused intravenously in doses varying between 0 1 γ to 3 γ/kg body weight/min. According to CELANDER (1954) these doses of catechol amines are comparable to the amounts secreted by the adrenal glands when the splanchnic nerves are activated at physiological frequencies. The effects obtained by the different doses of adrenaline on the jejunal and ileal motor activity were compared with special regard to eventual quantitative differences in the extent of the inhibition. In a few experiments the effect of vagal stimulations on the jejunal and the ileal motor activity was also studied while the intestine was under the influence of adrenaline in order to find out whether its depressing effect on the vagal excitatory responses was stronger in one part of the intestine than in another. These experiments were performed in cats in whom vagal stimulations in the neck induced motor responses in both the jejunum and the ileum of approximately the same magnitude.

In Fig 7 the jejunal and ileal responses induced by increasing intravenous doses of adrenaline are illustrated. In this experiment intestinal inhibition was obtained in both jejunum and ileum with 0 3 γ/kg/min with a simultaneous small increase of the blood pressure and a hardly noticeable decrease of the intestinal blood flow. Higher doses of adrenaline did not markedly enhance

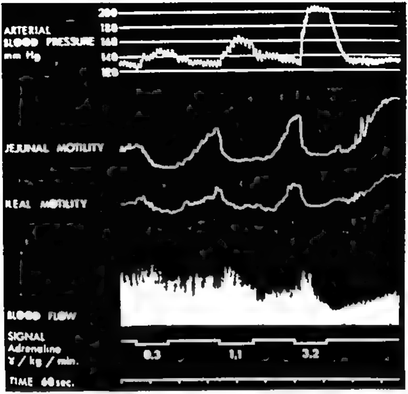

Fig 7 Cat 3 2 kg Chloralose urethane Effects of intravenous infusions of l adrenaline in successively increasing amounts on jejunal and ileal motor activity

Both vagal nerves were cut in the neck The adrenal glands were ligated The splanchnic nerves were intact Resting intestinal blood flow 38 ml/min/100 g intestinal tissue

the inhibitory effects on either the jejunum or the ileum but the resistance to blood flow within the intestine as well as the blood pressure increased in a graded fashion It thus appears that in this experiment a nearly maximal inhibition of intestinal motility was obtained in both the jejunum and the ileum with 0 3 γ of adrenaline/kg/min a dose which only slightly affected the blood pressure

In general also threshold inhibitory responses in the jejunum and the ileum appeared at largely the same concentrations of adrenaline in these experiments and were usually of the same magnitude at suprathreshold concentrations There was no evidence for any significantly higher sensitivity of the ileum to adrenaline in any of the experiments This does not exclude the fact that it was always possible to induce more extensive inhibitory responses with a suprathreshold dose of adrenaline if in any of the segments intestinal tone was initially high simply because the relaxation can then always be far more drastic than if tone is initially low

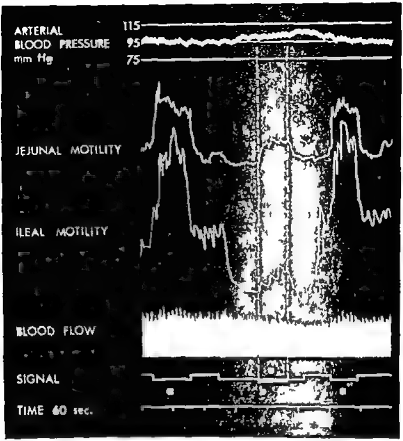

Fig 8 Cat 2 9 kg Chloralose urethane Effects of efferent cervical vagal stimulation (4 imp /sec 4 0 msec and 8 V) before (a) during (a) and after (a) intravenous infusion of l adrenaline 0 4 γ/kg /min on jejunal and ileal motility and blood flow Note that intravenously infused adrenaline suppressed the vagally induced motor responses in jejunum and ileum to approximately the same extent

Both vagal nerves were cut in the neck The adrenal glands were ligated The splanchnic nerves were intact Resting intestinal blood flow 45 ml/min/100 g intestinal tissue

Fig 8 shows an experiment in an adrenalectomized cat in whom the right vagus was stimulated in the neck with supramaximal intensity and 4 imp /sec at *a* Motor responses of approximately the same magnitude were obtained in both jejunum and ileum In b 0 4 γ of adrenaline/kg body weight/min was infused intravenously which induced a small inhibition in jejunal motor activity but a greater inhibition of the ileal motor activity During this infusion of adrenaline the vagal stimulation was repeated (*a*) now inducing reduced motor responses in both jejunum and ileum but the reduction of the responses

was of roughly the same degree in the two sections The infusion of adrenaline was then interrupted and the vagal nerve again stimulated (a'') which elicited motor responses of the same magnitude as during the first stimulation — In none of these experiments did adrenaline suppress the vagal influence on the ileum more than on the jejunum Infusions of large doses of adrenaline 1 to 2 γ/kg/min usually completely prevented the vagally induced motor responses in both the jejunum and the ileum The more intense the stimulation with respect to frequency the higher the dose of adrenaline needed to prevent the vagal excitatory responses but there was never observed any significant difference between the jejunum and the ileum in this respect

C Effects of stimulation of the splanchnic nerves on jejunal and ileal motility

Experiments were performed on 15 acutely vagotomized cats in whom both adrenal glands were excluded from the circulation without interference with the sympathetic nerve supply to the intestine Blood pressure jejunal and ileal motor activity and blood flow were recorded in the usual way The left splanchnic nerves were cautiously dissected free either in the thorax or in the abdomen The nerves were cut and the distal ends put on an electrode The right splanchnic nerves were left intact in these experiments except in four animals in whom the nerves were dissected free on both sides and cut centrally and all four nerves stimulated simultaneously as described in Chapter II The nerves were excited with supramaximal stimuli and the impulse frequency was varied between 2 and 20 imp /sec

Stimulation of the splanchnic nerves at low frequencies 2—6 per sec elicited prompt inhibitions of the *ileal* motor activity in 14 out of the 15 experiments but not so in the jejunum These promptly occurring ileal inhibitions appeared simultaneously with the onset of the intestinal vasoconstrictor responses In six experiments small inhibitory responses were seen in the jejunum and then only after a delay of about 20—30 seconds It was necessary to raise the stimulation frequencies to supraphysiological rates i e above 8—10 imp /sec in order to produce immediate and clearcut inhibitory responses in the jejunum as well, confirming the results of CELANDER (1959) and KOCK (1959) As a consequence of the vasoconstrictor fibre activation the intestinal blood flow resistance increased initially some 2—5 times when physiological frequencies of increasing rates were applied to the splanchnic nerves on both sides On unilateral splanchnic stimulation the regional flow resistance increased somewhat less for any given frequency

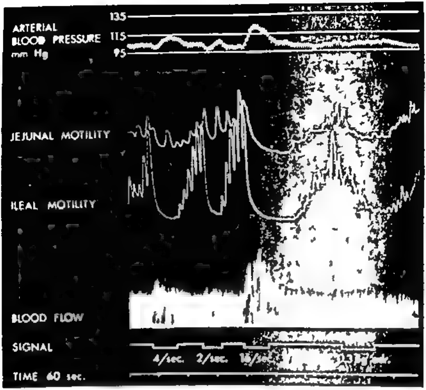

Fig 9 Cat 3 1 kg Chloralose urethane Effects of efferent stimulations of the left splanchnic nerves (10 V 4 0 msec) at different impulse rates and of intravenous infusion of adrenaline 0 3 γ/kg/min on jejunal and ileal motility and blood flow Note that splanchnic nerve stimulation at low physiological frequencies elicits immediate and intense inhibitions of the ileal motility but no significant changes of the jejunal motility Note also that splanchnic nerve stimulation at a high frequency and intravenous infusion of adrenaline induce almost the same inhibitory responses in jejunum and ileum

Both vagal nerves were cut in the neck The adrenal glands were ligated The right splanchnic nerves were intact Resting intestinal blood flow 90 ml/min/100 g intestinal tissue

A typical experiment is illustrated in Fig 9 Stimulation of the left splanchnic nerves with 4 and 2 imp /sec induced immediate inhibitions of the ileal motor activity without any promptly occurring significant changes of the jejunal motor activity Simultaneously with the ileal inhibitions small intestinal vasoconstrictor responses were obtained Splanchnic nerve stimulation with 16 imp /sec elicited prompt intestinal inhibitions in both the jejunum and the ileum at the same time that the intestinal blood flow decreased to about half the initial value 0 3 γ of adrenaline/kg body weight/min was then infused intravenously into the lower caval vein This dose caused a clearcut inhibition of both jejunal and ileal motor activity after a latency of 20 seconds without any significant effect on the intestinal blood flow

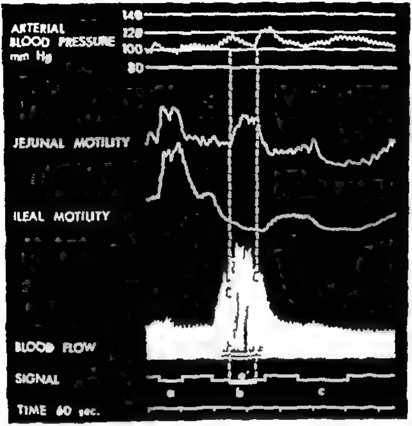

Fig 10 Cat 2·7 kg Chloralose urethane Effects of bilateral efferent cervical vagal stimulation (6 imp /sec 8 V and 4 0 msec) before (a) and during (a′) bilateral efferent splanchnic nerve stimulation (b) (6 imp /sec 12 V and 3 0 msec) on jejunal and ileal motility and of intravenous infusion of adrenaline 0 3 γ/kg/min (c) Note that splanchnic nerve stimulation induced inhibition of the ileal tone only Note also that a vagal motor response is obtained only in the jejunum during simultaneous splanchnic nerve stimulation

Both vagal nerves were cut in the neck The adrenal glands were ligated Resting intestinal blood flow 100 ml/min/100 g intestinal tissue

In a few experiments the vagi were stimulated during a prevailing activation of the splanchnic nerves The respective nerves were then excited at supramaximal intensities so as to activate all the fibres and the frequencies used were all within the physiological range These experiments were performed in order to find out the mutual relationship between the effects exercised by these two sets of nerve fibres on the ileal motor activity as compared with the jejunum Fig 10 shows such an experiment The two adrenal glands were excluded by ligatures and the animal was prepared as previously described Supramaximal stimulation of the right vagal nerve in the neck with

6 imp/sec induced increased motor activity in the two parts of the intestine as illustrated at *a* in Fig 10 At *b* the splanchnic nerves were stimulated with 6 imp/sec which caused a prompt inhibition of the ileal tone without any noticeable change of the jejunal motor activity While the splanchnic stimulation was still going on the vagal stimulation was repeated (*a'*) the stimulation characteristics being the same as in *a* Essentially the same motor response was obtained in the jejunum but in the ileum there was no sign of any increased motor activity Finally 0 3 γ of adrenaline/kg of body weight/min was infused intravenously (*c*) which induced inhibition of both jejunal and ileal motor activity indicating that the absence of an inhibitory response in the jejunum to splanchnic nerve stimulation could not be ascribed to any lack of intestinal tone It was not always possible to abolish entirely the vagally induced motor responses in the ileum by a simultaneous splanchnic nerve stimulation as factors such as stimulation frequency etc evidently affected the extent of the opposing vagal and splanchnic influences

To summarize the experiments in this section have shown that splanchnic nerve stimulation with physiological frequencies induced prompt inhibitions of the ileal motor activity Such inhibitory responses were not obtained in the jejunum until the splanchnic nerves were stimulated at supraphysiological frequencies Furthermore splanchnic nerve stimulation prevented completely or reduced vagally induced motor responses in the ileum but not to any significant extent in the jejunum

D Effects of vagal stimulation in the neck on jejunal and ileal motility before and after administration of guanethidine or ergotamine

The purpose of the experiments in this section was to find out if the ileum might be exposed to a tonic inhibitory influence via the splanchnic nerves which by its suppressing influence on the vagal excitatory effect explains the lack of vagal influence on the ileum The effect of vagal stimulation in the neck on the jejunal and ileal motor activity was therefore compared with intact splanchnic nerves before and after the influence of the splanchnic supply on the small intestine had been blocked by guanethidine or ergotamine Jejunal and ileal motor activity blood pressure and intestinal blood flow were recorded in nine acutely vagotomized and adrenalectomized cats chosen from those animals in whom cervical vagal stimulation elicited good motor responses in the jejunum but no or only minor motor responses in the ileum After the two vagal nerves had been repeatedly stimulated in the neck with supramaximal impulses at physiological frequencies 0 2—2 mg/kg of

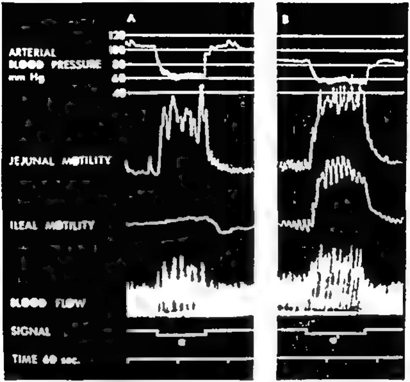

Fig 11 Cat 2 8 kg Chloralose urethane Effect of efferent bilateral vagal nerve stimulation (6 imp /sec 10 V and 2 0 msec) before (a) and after (a) intravenous infusion of ergotamine 0 5 mg/kg on jejunal and ileal motility Note that the ileal motor responses with cervical vagal stimulation is strongly enhanched by administration of ergotamine

Both vagal nerves were cut in the neck The adrenal glands were ligated The splanchnic nerves were intact Resting intestinal blood flow 8_ ml min/100 g intestinal tissue

guanethidine or 0 4—0 6 mg/kg of ergotamine was injected intravenously and the vagal nerves were again stimulated with the same stimulation parameters

Fig 11 illustrates a typical experiment In panel *A* the two vagal nerves were stimulated simultaneously with 6 imp /sec 10 V and 2 msec which elicited a strong motor response in the jejunum but a very slight response in the ileum Between panel *A* and *B* in the figure 0 5 mg/kg of ergotamine was injected intravenously in order to block a possible tonic sympathetic influence on the small intestine The vagal stimulation was then repeated (panel *B*) using the same stimulation parameters and induced now increased motor responses which were almost identical in magnitude in the jejunum and the ileum Essentially the same results were obtained in all the nine experiments of this series Only occasionally did sympathetic block increase the vagal effect on the jejunum as well and then never more than to result in

jejunal and ileal motor responses of approximately equal magnitude The present findings were further confirmed in some of the experiments described in detail in the next chapter where for other purposes the vagal nerves were stimulated below the diaphragm before and after the sympathetic innervation to the small intestine was blocked by guanethidine or spinal anaesthesia

To summarize these experiments indicate that the interruption of a tonic sympathetic influence causes the initially very weak ileal motor responses to vagal stimulation to become as intense as those of the jejunum

E Comments

The purpose of the experiments in this chapter was to investigate why vagal stimulation in the neck did not induce motor responses in the ileum as regularly as in the jejunum (see Chapter III) Several mechanisms may be involved such as regional differences in sensitivity to acetylcholine and catecholamines and/or in the distribution of excitatory vagal fibres and inhibitory sympathetic fibres

If this difference in motor responses is due to a difference in sensitivity of the jejunum and the ileum to acetylcholine it would appear also when this drug is administered intraarterially It would express itself as a lower threshold and larger motor responses to submaximal concentrations in those parts of the intestine which are more sensitive to the transmittor From the experiments presented in section (A) of this chapter it seems unlikely that the observed differences between the jejunal and ileal motor responses upon vagal stimulation could be due to any difference in the sensitivity of the smooth muscles to the cholinergic transmittor

No significant difference in catechol amine sensitivity was observed with intravenous infusion of adrenaline This was the case both in those experiments in which the jejunal and ileal responses to vagal stimulation were similar and those in which they differed Only in those experiments in which the two segments differed markedly with respect to the prevailing level of intestinal tone did suprathreshold concentrations of adrenaline cause a proportionally greater inhibition in the more active segment but here too there was no significant difference in threshold sensitivity Differences of this nature seem to reflect merely the fact that a smooth muscle inhibitory response will usually appear more striking in extent with a higher preexisting tonic motor activity

The absence of any significant difference in sensitivity to catechol amines between the jejunum and ileum was further supported by those experiments in which graded vagal nerve stimulations produced motor responses in the ileum as great as those in the jejunum In these cases when increasing amounts

of adrenaline were infused during a vagal activation of the two segments it was observed that the motor responses were depressed to essentially the same extent until at high adrenaline levels the vagally induced responses were completely suppressed at essentially the same adrenaline concentrations in both segments. These findings when taken together make it unlikely that the observed differences between jejunal and ileal responses to vagal stimulation in the neck were due to any difference in sensitivity of the jejunum and the ileum to adrenaline.

In the third part (C) of the experiments described in this chapter the effects of graded splanchnic nerve stimulation were studied with respect to possible differences in the influence on jejunal and ileal motor activity. The secretion from the adrenal medulla had been excluded so that the responses could be ascribed solely to the nerves. The present results agree with the observations of CELANDER (1959) and KOCK (1959) in so far as the jejunum does not seem to be supplied with inhibitory fibres from the splanchnic nerves. It was observed, however, that immediate inhibitory responses could be obtained in the ileum when the splanchnic nerves were stimulated at low frequencies well within the physiological discharge rate. These prompt ileal inhibitions can hardly be ascribed to effects of transmittor overflow from the vasoconstrictor fibre nerve endings as such an overflow reaches concentrations of significance for adjacent, normally sensitive effectors only at supraphysiological frequencies (CELANDER 1954, BROWN and GILLESPIE 1957).

The possibility remains that these ileal inhibitions are secondary to the blood flow decrease caused by the activation of the ileal vasoconstrictor fibres, a factor which no doubt can interfere with intestinal motor activity in general. However, according to KOCK (1959) the intestinal blood flow generally has to be reduced as much as to 1/3—1/4 of the resting value before a blood flow restriction *per se* induces a clearcut intestinal inhibition. Furthermore, such a type of intestinal inhibition is relatively delayed in onset as compared with direct nerve fibre influences and is often preceded by a transient increase of motility (CELANDER 1959, obvious also from Fig. 15 in KOCK's paper 1959). In most of the present experiments low frequency splanchnic stimulation caused such relatively weak vasoconstrictor effects that the blood flow reduction *per se* cannot explain the immediate ileal inhibitions obtained, the more so as considerably more drastic mechanical obstructions of ileal blood flow did not cause any significant promptly beginning ileal inhibitions. It is therefore not likely that these ileal inhibitory responses to low frequency splanchnic stimulation were secondary either to the concomitant blood flow decrease or to any overflow of the adrenergic transmittor.

On the basis on these findings it therefore seems justified to conclude that these sympathetically induced ileal inhibitions are caused by specific inhibitor fibres distributed to this section of the small intestine. Likewise the absence of significant inhibitory responses in the jejunum favours the view that the splanchnic nerves carry few, if any, fibres to proximal parts of the small intestine.

It was observed incidentally in a number of adrenalectomized cats with intact splanchnic nerves that the centrally conveyed reflex increase of sympathetic activation, induced by carotid occlusion, did not produce any jejunal inhibition, in this respect confirming the findings of KOCK (1959). The same procedures, however, induced frequently a prompt inhibition of ileal motility. These findings, too, suggest that there is no, or at least only a very sparse, splanchnic distribution of specific inhibitory fibres to the jejunum which can be centrally excited from bulbar centres, while the ileum seems to be fairly richly innervated in this respect.

It could be shown in animals with sectioned splanchnic nerves that in the ileum, but not in the jejunum, vagally induced motor activity could be prevented completely or reduced by simultaneous low frequency splanchnic stimulation. Thus, if ileal motor activity can be fairly selectively suppressed by tonic activity of splanchnic inhibitory fibres, such a mechanism alone may be responsible for the fact that vagal stimulation either did not effect or only slightly enhanced the ileal motor activity in many of the present experiments. If this is so it would be expected that equally strong motor responses in both the jejunum and the ileum should be obtained upon vagal stimulation after blocking the sympathetics. This possibility was tested in the last part in this chapter (section D). Here it was shown that vagal stimulation after blocking the sympathetic innervation to the small intestine with guanethidine or ergotamine induced approximately equal motor responses in the jejunum and the ileum in those experiments in which there were clearcut jejunal motor responses but absent or minimal ileal responses before the administration of these drugs. It therefore seems justified to conclude that at least in some patterns of increased sympathetic discharge an activation of specific inhibitory fibres occurs, which affect essentially only ileal sections of the small intestine. If fibres of this type are distributed to the jejunum they must be few and of little significance for jejunal motor activity.

Thus, it seems that the tonic activity of such centrally governed inhibitory fibres to the ileum accounts for the suppression of vagal excitatory influence on the ileum in about half of the present experiments. Furthermore, it seems justified to conclude from the present experiments that the whole small intestine is supplied with vagal motor fibres to approximately the same extent

CHAPTER V

Effects of subdiaphragmatic and intrathoracic vagal stimulation on the jejunal and ileal motility and blood flow

From histological studies of the vagal nerves in cats it is known that they are not admixed with any sympathetic fibers at the cervical level. However below the level of the heart there is an admixture of such fibres (HEINBECKER and O'LEARY 1933; RANSON, FOLEY and ALPERT 1933; MOHIUDDIN 1953; EVANS and MURRAY 1954 and AGOSTONI *et al* 1957). According to HILLARP (1960) the destination and function of this sympathetic admixture to the vagal nerves are still not known. Earlier studies have not given any convincing proof of the presence of intestinal inhibitory fibers in the subdiaphragmatic or intra thoracic portions of the vagal nerves.

Furthermore, it has not been definitely settled whether the vagal nerves contain any parasympathetic vasodilator fibers to the vessels of the small intestine (McDOWALL 1935; FOLKOW 1955). According to CELANDER and FOLKOW (1951) it will be difficult to reveal the existence of such fibers since the intestinal smooth muscles will probably be activated simultaneously upon vagal stimulation. In that case the increased intestinal motor activity could interfere mechanically so much with the intestinal blood flow that it could mask a neurogenic vasodilator effect. On the other hand an increased motor activity as well as a neurogenic secretory response might also cause meta bolite accumulation which *per se* could produce vasodilation and thus simulate a truly neurogenic vasodilator response. However, there does not seem to be any systematic investigation in which both intestinal motor activity and blood flow have been recorded simultaneously during graded vagal stimulations in order to explore whether or not any specific vasodilator fibres exist.

In the experiments described in this chapter vagal stimulations have been performed below the heart level partly in order to avoid concomitant changes in pressure head with its secondary effects on the intestinal blood flow and partly to allow for an excitation of any sympathetic fibres joining the vagal nerves in the lower thoracic region. It would then be possible to explore whether those sympathetic fibres present affect intestinal motility and blood flow. Also by such stimulations it would be easier to reveal whether any specific vagal vasodilator fibres are distributed to the intestine.

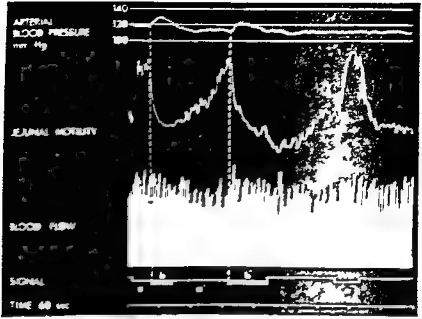

Fig. 12. Cat 2.2 kg. Chloralose-urethane. Effects of "weak" (4 imp/sec 8 V and 1.0 msec) and "strong" (4 imp/sec 8 V and 6.0 msec) efferent stimulations of the right vagal nerve at the subdiaphragmatic level on jejunal motility and blood flow. Note that stimulations with "weak" currents (a, a' and a") induce excitatory responses and stimulations with "strong" (b and b') currents inhibitory motor responses.

Both vagal nerves cut in the neck. The adrenal glands and the splanchnic nerves were intact. Resting intestinal blood flow 44 ml/min/100 g intestinal tissue.

A. Results

Forty cats were used in this series of experiments. In thirty animals the vagal nerves were carefully dissected free from adjacent tissues immediately below the diaphragm and cut as far proximally as possible. For technical reason only one of the nerves at a time was stimulated, in most cases the right (posterior) one as it usually proved impossible to influence intestinal motor activity by left vagal stimulation at this low level. The jejunal motor activity, the blood pressure and the intestinal blood flow were recorded in all thirty animals. In thirteen of these the ileal motor activity was recorded as well. In ten additional animals the vagal nerves were stimulated at the lower thoracic level as described in Chapter II. In all of the experiments the vagal nerves were stimulated with 4 or 8 imp/sec while voltage and pulse-duration were varied within wide limits (4—20 V, 1—6 msec). Before stimulation the vagal nerves were cut in the neck. Initially the splanchnic nerves and the adrenal glands were left intact.

a) *Intestinal motility* Fig 12 shows an experiment in which the right vagal nerve was stimulated below the diaphragm during registration of the jejunal motor activity and intestinal blood flow At *a* the nerve was stimulated with 8 volts 1 msec and 4 imp /sec which induced an almost immediate increase of the jejunal motor activity with no noticeable effect on blood pressure and intestinal blood flow Without interruption of stimulation the pulse duration was then increased to 6 msec (*b*) which elicited an immediate inhibition of the jejunal motor activity and simultaneously the blood pressure increased about 10 mm Hg while the jejunal blood flow in this case at least remained practically unchanged This was rather the exception than the rule as will be outlined below Still *without interruption of the stimulation the pulse* duration was shifted back to 1 msec (*a*) and now the jejunal motor activity increased once more though not as promptly as with the first stimulation When the pulse duration was again increased to 6 msec (*b*) the jejunal motor activity once more was promptly inhibited and the blood pressure increased as *in b* The vagal stimulation was then interrupted *for a few minutes* and started again at a with the same parameters as at *a* and [illegible] resulting in a strong and prompt jejunal motor response showing that the excitatory fibers were still intact after the periods of intensified stimulation The inhibitory responses at *b* and *b* cannot have been due to damage or failing activity of the excitatory fibers but were more likely induced by a specific set of high threshold fibers the more so as there was a simultaneous increase in blood pressure

It was also possible to inhibit the jejunal motor activity by vagal stimulation without first inducing an increased motor activity as in Fig 12 This could be accomplished by using a higher pulse duration or voltage initially (cf e g Fig 14 panel *B b*) Simultaneously with these intestinal inhibitions the intestinal blood flow usually decreased and the blood pressure increased which is also illustrated by this part of Fig 14 In a few experiments these vagally induced intestinal inhibitions were not maintained with continued vagal stimulation but either shifted over gradually to an increased motor activity or the intestinal tone returned to the prestimulatory level However in only five of the thirty experiments in which subdiaphragmatic vagal stimulations were performed did the typical pattern of jejunal inhibition increased blood pressure and decreased intestinal blood flow fail to appear with high intensity vagal stimulation

In those experiments in which the ileal motor activity was recorded as well low voltage vagal stimulation elicited excitatory responses in the ileum Here also clearcut inhibitory responses were induced when voltage and/or pulse duration were increased to the same level that produced jejunal inhibitory

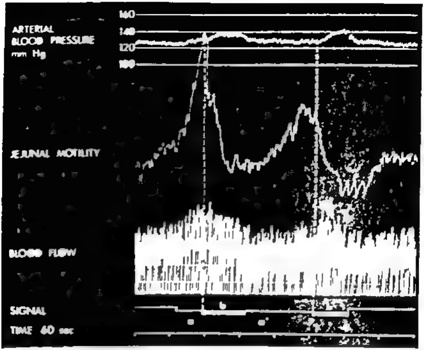

Fig 13 Cat 3 7 kg Chloralose urethane Effects of "weak" (4 imp/sec 8 V and 2 0 msec) and "strong" (4 imp/sec 8 V and 8 0 msec) efferent stimulations of the left intrathoracic vagal nerve on the jejunal motility and blood flow Note that stimulations with weak currents (a and a') induce excitatory responses and stimulations with strong (b and b') currents inhibitory motor responses Note also that the blood pressure increases and the blood flow decreases simultaneously with the inhibitory motor responses

Both vagal nerves were cut in the neck. The adrenal glands and the splanchnic nerves were intact "Resting" intestinal blood flow 26 ml/min/100 g intestinal tissue

responses It was then generally noticed that the ileal inhibitions if anything were more pronounced than those of the jejunum. Further the ileal motor activity never increased again during a prevailing inhibitory vagal stimulation as was sometimes the case with the jejunum

In order to exclude the possibility that the intestinal inhibitions and vascular responses obtained with subdiaphragmatic vagal stimulation were due merely to a spread of current to adjacent afferent fibres e g in the peritoneum thus eliciting the so-called peritoneo intestinal inhibitory reflex (KING 1924 DOUGLAS and MANN 1941) in ten cats the vagal nerves were dissected free and stimulated at the lower thoracic level the diaphragm being entirely intact In nine of these animals intrathoracic vagal stimulation induced excitatory as well as inhibitory responses in the jejunum combined with a blood pressure rise and an intestinal vasoconstriction, all the responses appearing at

the same stimulus parameters as those used for subdiaphragmatic stimulation Fig 13 illustrates such an experiment At *a* the right vagal nerve was stimulated with 4 imp/sec 8 V and 2 msec The jejunal motor activity increased The blood pressure rose slightly and there was a small decrease in the blood flow Without interruption of the stimulation the pulse duration was increased to 8 msec (*b*) The jejunal motor activity was promptly inhibited and the blood pressure increased further about 10 mm Hg with no noticeable change in the blood flow At *a* and *b* the same procedures were carried out as at *a* and *b* still without interruption of the stimulation

It is thus obvious that stimulation of the vagus in the neck differs from subdiaphragmatic or intrathoracic stimulation since distinct intestinal inhibitory responses intestinal vasoconstriction and pressor effects can be induced from the latter sites of stimulation This difference is further illustrated in fig 14 Here the right vagal nerve was stimulated first in the neck (*A*) and then below the diaphragm before (panel *B*) and after (panel *C*) administration of guanethidine At *a* in panel *A* the stimulation parameters were 4 imp/sec 4 V and 2 msec causing increased motor activity in the jejunum with very slight motor responses in the ileum These responses did not change when the stimulus strength was increased to 8 V (*b*) No vasodilator response is seen and if anything intestinal blood flow resistance increased slightly at both stimulations The right vagal nerve was then stimulated just below the diaphragm after having been cut centrally Panel *B* demonstrates the effect of this stimulation where exactly the same stimulation parameters were used in *a* and *b* as in *a* and *b* of panel *A* The lower stimulation intensity (*a*) induced virtually the same jejunal and ileal responses as in *a* of panel *A* No vasodilator response is seen but the vagal depressor influence on the heart and consequently on the blood pressure is now of course missing In *b* of panel *B* the stimulus strength was increased to 8 V just as in (*b*) of panel *A* but now the vagal stimulation elicited an almost immediate and equally marked inhibition of both jejunal and ileal motor activity Simultaneously the blood pressure increased about 40 mm Hg and intestinal blood flow decreased Thus the same stimulus strength which induced intestinal excitation when applied to the vagal nerve at the neck level induced quite the opposite intestinal responses when applied to the nerve at the subdiaphragmatic level In addition intestinal blood flow now decreased and blood pressure rose It should also be noted that these excitatory and inhibitory motor responses to vagal stimulations were induced from the same level of intestinal tone Between panel *B* and *C* 2 mg/kg of guanethidine was injected intravenously to block possible adrenergic fibers The vagal nerve was then again stimulated subdiaphragmatically with the stronger current (*b* in panel *C*) which earlier

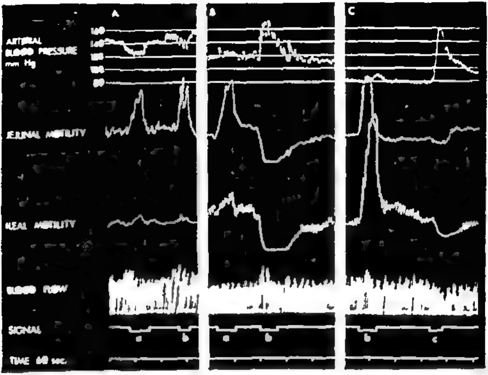

Fig. 14. Cat 3.1 kg. Chloralose-urethane. Effects of efferent vagal stimulation at the cervical and subdiaphragmatic levels on jejunal and ileal motility and blood flow and the blood pressure. In A the right vagal nerve is stimulated in the neck with a comparatively weak (4 imp./sec., 4 V and 2 msec) and strong (b) current (4 imp./sec., 8 V and 2 msec). In B the right vagal nerve is stimulated below the diaphragm with the same stimulus strength as in A, and in C at the same level with the stronger current after administration of guanethidine (2 mg/kg.). In c of panel C adrenaline 0.7 γ/kg was given intravenously. Note that inhibitory responses are induced with subdiaphragmatic stimulation with the "stronger" current while subdiaphragmatic stimulation after administration of guanethidine and cervical vagal stimulation with the same stimulus strength induces excitatory responses. Note also the difference between the ileal motor responses with vagal stimulation before and after administration of guanethidine.

Both vagal nerves were cut in the neck. The adrenal glands and the splanchnic nerves were intact. "Resting" intestinal blood flow 36 ml/min/100 g intestinal tissue.

induced prompt intestinal inhibition, intestinal vasoconstriction and blood pressure rise. Now an increased motor activity was obtained in *both* jejunum and ileum while there was no longer any clearcut effects on blood pressure or on intestinal blood flow, certainly no intestinal vasodilatation was induced. On the whole, it was impossible to induce any inhibitory responses after administration of adrenergic blocking drugs such as guanethidine even if the stimulation parameters were changed over a wide range. Note further that vagal stimulation elicited a stronger ileal motor response after the administration of guanethidine. This is in agreement with the results described in section

D of Chapter IV and with the conclusions drawn from the results in that chapter — Finally 2γ of adrenaline was injected intravenously (c in panel C) which elicited inhibition of both jejunal and ileal motor activity indicating that the absence of inhibitory responses to vagal stimulation after guanethidine could not be ascribed to any lack of intestinal tone

In all the seven animals in whom guanethidine was injected after a clearcut and reproducible intestinal inhibitory response had been obtained by subdiaphragmatic vagal stimulation intestinal inhibitory responses could no longer be induced by electrical stimulation The blood pressure increase and blood flow reductions were also largely abolished Instead jejunal and ileal motor responses of largely equal magnitude were now obtained with the same stimulation parameters that before administration of guanethidine had elicited prompt inhibitory responses In no case was intestinal vasodilatation observed as a response to the vagal stimulation

In the experiments presented above the splanchnic nerves were as previously mentioned initially left intact In six of the experiments the splanchnic nerves were cut after it had been shown that repeated subdiaphragmatic vagal nerve stimulations induced clearcut inhibitory responses In two additional animals spinal anaesthesia was given in order to block any prevailing sympathetic activity to the small intestine Repetition of the same vagal stimulation now failed to induce intestinal inhibitory responses in all eight animals Instead intestinal excitation occurred and the previous blood pressure increases and intestinal blood flow reductions disappeared or were markedly reduced Thus it seems evident that the splanchnic nerves and their spinal medullary connections had to be intact for the inhibitory intestinal responses to subdiaphragmatic vagal stimulation to appear

In most experiments the results of subdiaphragmatic vagal stimulation at high intensity were compared with the so-called intestino intestinal inhibitory reflex This was elicited by afferent stimulation of the periarterial nerves in a mesenteric pedicle and equally pronounced reflex inhibition of the jejunum and the ileum were performed In contrast to the vagal inhibitory response this reflex remained largely unimpaired after splanchnic nerve section but like the vagal inhibitory response it disappeared after spinal anaesthesia or after administration of guanethidine

b) *Intestinal blood flow* In the present experiments great care was taken to investigate the effect of subdiaphragmatic vagal stimulation on the intestinal blood flow in order to find out if the vagal nerves convey specific parasympathetic vasodilator fibres to the vessels of the small intestine With this low level stimulation the disturbing depressor effects obtained with vagal stimulation in the neck can be avoided In thirty animals more than five

hundred separate stimulations were performed with square wave pulses at a constant frequency usually 4 or 6 imp /sec while voltage and pulse duration were varied within wide limits In many series of stimulations the voltage was also changed over a wide range while the pulse duration was kept constant No one of these different subdiaphragmatic vagal stimulations induced any intestinal vasodilator responses but only excitatory or inhibitory motor responses as described above Strong currents however elicited intestinal vasoconstrictor responses which disappeared after administration of guanethidine as previously mentioned Repetition of the same vagal stimulation after injection of this drug did not produce any vasodilator responses while intraarterially injected acetylcholine in small amounts readily produced intestinal vasodilatation The vagal nerves were also stimulated in the thorax below the heart level in ten animals The effect on the intestinal blood flow in these experiments was the same as obtained with subdiaphragmatic vagal stimulation i e vasodilator responses were never seen The present experiments have thus shown that no noticeable intestinal vasodilator responses can be induced by subdiaphragmatic or intrathoracic vagal stimulation nor were vasodilator responses ever obtained when the vagal nerves were stimulated at the neck level

To summarize the results of these experiments have shown that it is possible to induce not only intestinal excitatory but also intestinal inhibitory responses if the vagal nerves are stimulated at the subdiaphragmatic or intrathoracic level These inhibitory responses appeared regularly when the stimulus strength was raised above a certain threshold level well above that needed to stimulate the excitatory fibres These inhibitory responses tended to be somewhat more pronounced in the ileum and they were accompanied by intestinal blood flow reductions and blood pressure increases The circulatory responses as well as the intestinal inhibitory responses disappeared however after abdominal splanchnic nerve section after spinal anaesthesia or after administration of guanethidine When compared with the so called intestino intestinal inhibitory reflex in the same experiment this latter reflex also disappeared after spinal anaesthesia or administration of guanethidine but it remained largely unchanged after abdominal splanchnic nerve section The intestino intestinal inhibitory reflex usually produced equally extensive inhibitions of both the jejunum and the ileum

Furthermore vasodilator responses could never be induced by subdiaphragmatic or intrathoracic vagal stimulation

II Comments

The results obtained in this chapter differ from those obtained with vagal stimulation in the neck (see Chapter III) in so far as distinct inhibitory motor responses could not be produced by stimulations at this level even when intensities were used which by far exceeded those which at the subdiaphragmatic or lower thoracic level induced intestinal inhibition and vasoconstriction

It might be suggested that the intestinal inhibitory responses were due to an accidental activation of the so called peritoneo intestinal inhibitory reflex caused by a spread of the stimulation current to adjacent afferent fibres This possibility was always considered during the subdiaphragmatic vagal preparations so that the nerves were carefully dissected free from adjacent tissues cut centrally and well isolated from adjacent tissues by means of a paraffin bath Furthermore intrathoracic vagal stimulations, above the intact diaphragm elicited inhibitory and excitatory motor responses in the same way that the subdiaphragmatic vagal stimulations did Therefore in view of the preparation and the results obtained with intrathoracic vagal stimulation it does not seem likely that the intestinal inhibitory response blood pressure rise and intestinal blood flow reduction could be due to an accidental activation of the so called peritoneo intestinal inhibitory reflex Furthermore since intestinal inhibitions could easily be induced by low frequency stimulation, these responses can hardly be explained as an effect of overflow of transmittor from concomitantly activated vasoconstrictor fibres as was discussed earlier in Chapter IV with reference to splanchnic nerve stimulation

It thus seems justified to conclude that the excitatory and inhibitory responses obtained with vagal stimulation at the lower thoracic and subdiaphragmatic levels must be ascribed to two different sets of nerve fibres Since vagal stimulation in the neck did not induce any distinct intestinal inhibitory responses this must imply that the vagal nerves receive a contribution of nerve fibres below the neck level having a higher stimulation threshold than the excitatory fibres

From histological studies of the vagal nerves in cats it is known as previously mentioned that thin efferent sympathetic fibers join these nerves in the thorax (for ref see HILLARP 1960) From the present experiments it seems likely at least at first sight that the intestinal inhibitory response the blood pressure increase and intestinal blood flow reduction obtained with vagal stimulation are to be ascribed to an activation of these sympathetic fibers the more so as these responses disappeared after administration of guanethidine Since these drugs block postganglionic adrenergic transmission but not cholinergic parasympathetic transmission (CASS and SPRIGG 1961 BOYD

GILLESPIE and MACKENNA 1962) it seems clear that the responses obtained are mediated by adrenergic nerve fibers

However the observation that splanchnic section or spinal anaesthesia abolished the intestinal inhibitory responses blood pressure increases and intestinal blood flow reductions are puzzling and make it questionable whether these responses really are directly induced by efferent sympathetic fibers joining the vagal nerves It would then have to be assumed that such fibers can significantly affect the effectors only when the end organ is simultaneously exposed to a discharge in splanchnic efferent fibers In other words a definite spatial summation would be needed to reveal their effector influence Spatial summation has been demonstrated in the peripheral autonomic system although hardly in such an all or none fashion as would have to be assumed to interpret the present findings to spatial summation

There is however another perhaps more likely possibility Experiments performed by VON EULER (1949) concerning the effects of galvanic antidromic stimulation of thin afferent vagal fibres may have some bearing on the present findings Pressor responses which were abolished by sympathectomy could be induced by galvanic stimulation of the vagal supply to the heart It was proposed that these pressor responses were due to a sympathetically mediated nociceptive reflex from the heart being activated by means of an antidromic excitation of thin afferent vagal fibers Sympathetically mediated nociceptive reflexes in all probability involving the so called intestino intestinal and peritoneo intestinal reflexes can readily be induced also from the gastrointestinal tract (e g YOUMANS 1944 GERNANDT and ZOTTERMAN 1946) Moreover both with respect to the characteristics of the response pattern and the effects of spinal anesthesia these reflexes closely mimic the effects induced by intrathoracic or subdiaphragmatic vagal stimulations in the present experiments Therefore in analogy with von Euler s suggestion these vagally induced responses may in fact be expressions of a nociceptive sympathetically mediated reflex being activated by an antidromic excitation by thin afferent fibres which had joined the vagal nerves at the lower thoracic level and therefore were excited only when vagal stimulations were performed at this level The presence of such afferent fibres has been described by HARPER MCSWINEY and SUFFOLK (1935) but has been denied by CRAGG and EVANS (1960) However at present it seems difficult to settle definitely to which of the above mentioned possible mechanisms the intestinal inhibitory and circulatory responses are to be ascribed or whether they might be due to still another type of mechanism

The question as to whether the vagal nerves convey parasympathetic dilator fibres to the blood vessels of the small intestine was also studied in the present

experiments. Thus the vagal nerves were stimulated below the diaphragm altogether more than five hundred times in thirty animals in which voltage, pulse duration and frequency were varied over a wide range. In no one of these stimulations did the intestinal blood flow resistance decrease as a result of vagal activation. This absence of vasodilator responses could not be dependent upon any simultaneous activation of vasoconstrictor fibres masking the effects of possibly existing vasodilator fibres since administration of guanethidine did not reveal any intestinal vasodilator responses on vagal stimulations. It could be suggested that the intestinal motor activity induced by vagal stimulation might interfere with the blood flow to such an extent as to conceal a concomitant neurogenic vasodilator response. Thus from experiments performed by e.g. BOATMAN and BRODY (1963) and confirmed in the present experiments, it is known that a strong motor activity induced by acetylcholine can cause a decrease in the intestinal blood flow which is evidently due to a compression and distortion of vessels lying within the intestinal wall. Such mechanical interferences were, however, seen only when large doses of acetylcholine were given. Lower concentrations readily dilated the intestinal vessels. With vagal stimulation, on the other hand, dilator responses were never seen in spite of the fact that obvious mechanical interferences with the blood flow were observed occasionally only when high frequency vagal excitations induced almost maximal intestinal motor responses.

With the wide variation in stimulation parameters used, it seems unlikely that the cholinergic excitatory fibres to the intestinal smooth muscles would be able to conceal systematically in some five hundred stimulations all expressions of concomitantly induced vasodilator responses, while such responses were so readily obtained when small doses of acetylcholine was administered via the blood stream. For such reasons it seems justified to conclude that the vagal nerves do not convey specific vasodilator fibres to the blood vessels of the jejunum and the ileum.

fashion It was then found that splanchnic nerve stimulation at physiological frequencies induced clearcut and largely equal vasoconstrictor responses in both the jejunum and the ileum and in addition significant and immediate ileal inhibition while no significant immediate jejunal inhibitions were seen This finding indicates a difference in the distribution of the splanchnic inhibitory fibres to the small intestine with a more abundant adrenergic innervation of the ileal section Furthermore, ileal motor responses induced by vagal stimulations could be prevented completely or reduced in extent by a simultaneous splanchnic nerve stimulation even when the adrenal glands were excluded which was not the case in the jejunum (section C)

In the last section (D) of Chapter IV the effects of vagal stimulation in the neck on jejunal and ileal motor activity were compared before and after the sympathetic nervous system had been blocked by guanethidine or ergotamine Animals in whom vagal stimulation elicited strong motor responses in the jejunum but no responses or only minor ones in the ileum showed equally strong motor responses in both the jejunum and the ileum when the same vagal stimulations were repeated after administration of one of these drugs From these findings it was concluded that the entire small intestine of the cat is supplied with vagal excitatory fibres to approximately the same extent The ileum however seems to differ from the jejunum insofar as it is exposed to the influence of tonically active inhibitory fibres in the splanchnic nerves From the present experiments it is not possible to settle exactly how far proximally the small intestine is supplied with this strong adrenergic innervation from the splanchnic nerves It should be stressed that the observations were carried out on isolated segments of ileum lying some centimeters proximal to the ileo coecal valve (see Chapter II) Thus the ileal inhibitory responses are separate from the function of the ileo coecal valve

In another series of experiments described in *Chapter I* the vagal nerves were stimulated also at the intrathoracic or subdiaphragmatic levels to investigate whether an admixture of sympathetic fibres to the vagal nerves at these levels (e g see HILLARP 1960) is of any importance for the control of the intestinal motor activity and blood flow Vagal stimulations at these levels at physiological frequencies and with low voltage and pulse durations elicited as was the case in the neck purely excitatory responses When however the stimulus strength was raised well above the threshold level of the excitatory fibres inhibitory effects on intestinal motility were obtained in both the jejunum and the ileum These inhibitory responses were accompanied by intestinal blood flow reductions and blood pressure increases These effects disappeared not only after administration of guanethidine but also after splanchnic nerve section or spinal anaesthesia Some conceivable mecha

nisms which could be in agreement with such a combination of effects were discussed One possibility is that thin afferent fibres were activated in the antidromic direction in one way or another causing an excitation of the intestino intestinal inhibitory reflex similar to that previously described for the vagal sympathetic innervation of the heart (v FULER 1949) Whatever the case it is not possible to conclude from the present experiment whether these responses are physiological or not

In the experiments described in Chapter V great care was also taken to investigate whether the vagal nerves contain any parasympathetic vasodilator fibres to the vessels of the small intestine For this purpose the vagal nerves were stimulated below the heart level to avoid the concomitant changes in blood pressure with its secondary effect on intestinal blood flow In no one of more than five hundred stimulations did the intestinal blood flow resistance decrease upon vagal stimulation although the stimulation voltage pulse duration and frequency were varied over a wide range Nor were any vasodilator responses obtained upon vagal stimulation after administration of guanethidine injected in order to exclude a possible masking effect of simultaneously activated vasoconstrictor fibres It therefore seems justified to conclude that the vagal nerves do not convey any specific vasodilator fibres to the intestinal blood vessels

The results of the present experiments may be criticized as being not necessarily representative of the intestinal responses occurring in an intact conscious animal However the electrical activity of the intestinal smooth muscles seems to be normal with urethane or chloralose anaesthesia (TEXTER 1964) and it is reasonable to suppose that the intestine is if anything less sensitive to nerve stimulations with operative procedures and anaesthesia than in the intact awake animal Most of the present experiments constituted a comparison between the effects of graded vagal and splanchnic nerve stimulations on the jejunal and ileal motor activity The results would therefore not be expected to be influenced qualitatively by such factors as the narcosis and the operative procedure especially as the jejunal and ileal motor activity was recorded simultaneously in most experiments

In previous investigations (see e g GARRY 1957 and VAN HARN 1963) two factors have been said to determine the direction of intestinal responses to vagal stimulation the state of the intestinal motor activity and the stimulation frequency Thus high tone and vivid motility was said to favour inhibitory responses and a low tone and weak motility excitatory motor responses Further low stimulation frequencies were said to favour excitatory and high frequencies inhibitory responses The results of the present experiments are not in agreement with these views for vagal stimulations either at the cervical

level or subdiaphragmatically HARPER *et al* (1959) MARTINSSON and MUREN (1963) and MARTINSSON (1964) were also unable to support such views suggested by earlier workers with respect to the gastric smooth muscles where instead it has been shown that the vagal nerves indeed carry both excitatory and inhibitory fibres with distinct differences in threshold (see MARTINSSON 1964) By changing the stimulus strength with subdiaphragmatic and intrathoracic vagal stimulation it was possible to produce excitatory as well as intestinal inhibitory responses at the same initial level of intestinal activity (see Fig 14 panel *B*) This does not deny that it was sometimes difficult to increase intestinal motor activity by vagal stimulation when it was very intense to start with just as it could be difficult to demonstrate clearcut inhibitions of intestinal motility when it was initially low However it is important to stress that while both excitatory and inhibitory responses could be elicited at any frequency used the direction of the response was clearly dependent only on the intensity of the stimulation i e on the voltage and pulse duration indicating the presence of two different sets of nerve fibres at these levels (for discussion of the nature of these fibres see Chapter V)

As previously mentioned HSU (1944) obtained intestinal inhibitory responses preceded by a few contractions with vagal stimulation at 50 imp /sec It is possible that such inhibitory responses may simply be due to gradual failure of the transmission mechanism of the excitatory fibres being excited at such high supraphysiological rates (see Chapter III) rather than to a consequence of any activation of specific inhibitory fibres In general such an unspecific type of suppression might explain why high frequency stimulations have been considered to induce intestinal inhibitory responses

From the above mentioned facts it should be clear that it is important to use physiological frequencies and well defined intensities and pulse durations when the parasympathetic innervation to the small intestine is to be examined CELANDER (1959) and KOCK (1959) have both called attention to this with regard to splanchnic nerve investigations

In Chapter I it was pointed out that there are at least at first glance contradictory results concerning the motor responses of the upper and lower parts of the small intestine as a result of vagal stimulation Thus BAYLISS and STARLING (1899) observed in dogs increased motor activity in all parts of the small intestine upon vagal stimulation in the neck but only if the splanchnic nerves had previously been cut Likewise in rabbits in whom the spinal cord had been destroyed at the thoraco lumbar level which would also interrupt sympathetic impulses to the intestine vagal stimulation in the neck induced about equally strong motor responses throughout the various parts of the bowel (ALVAREZ *et al* 1929) KLEE (1912) and KURÉ *et al* (1931) on

the other hand stimulated the vagal nerves without any previous block of the sympathetic supply to the small intestine KLEE observed in cats that vagal stimulation in the neck usually had the most intense effect on the duodenum with weak or sometimes absent responses in the ileum KURÉ *et al* who stimulated the vagal nerves just above the diaphragm in dogs obtained motor responses throughout the small intestine in some animals but only in the upper parts in others These results are all in agreement with the present findings and can be explained by the fact that in the experiments of KLEE and KURÉ *et al* the splanchnic nerves were left intact That being the case activity in these nerves could suppress the vagal excitatory influence on the ileum as was shown in the present experiments The experiments of BAYLISS and STARLING and ALVAREZ *et al* correspond with the fact that equally strong motor responses in the jejunum and the ileum are obtained with vagal stimulation after the sympathetic influence to the small intestine has been blocked

The fact that no inhibitory motor re ponses can be obtained in the jejunum with splanchnic nerve stimulation with physiological frequencies does not imply (as mentioned in Chapter I) that the jejunum lacks entirely sympathetic inhibitory fibres It should be remembered that in addition to the above mentioned bulbar sympathetic control of intestinal motility there exists a strictly spinal inhibitory system as well often called the intestino intestinal inhibitory reflex (cf JOHANSSON and LANGSTON 1964) and this type of reflex too is blocked by adrenolytic drugs and by spinal anaesthesia Whether it operates by way of the same efferent links as the centrally controlled adjust ments is not known but it differs from the central adjustments not only by the fact that it is activated only via abdominal visceral afferents but also in the important respect that it causes a prompt and equally profound inhibition of the jejunum as well as of the ileum The presence of this spinal reflex necessitates the assumption that efferent inhibitory fibres are distributed to the jejunum also though the engagement of these fibres seems to be largely confined to this type of regional reflex It remains to be shown exactly where the efferent fibres involved run at least with respect to the jejunum The absence of immediate jejunal inhibitory responses to direct splanchnic stimu lation suggests that they may run along other nerve connections between the spinal medulla and the intestine a view which is in agreement with the fact that jejunal intestino intestinal reflexes can still be elicited after section of all the splanchnic nerves (KUNTZ and SACCOMANNO 1944 KOCK 1959) It is very likely that this spinal reflex mechanism has often been activated to a greater or lesser degree in experiments in which there is unavoidable handling and traumatization of abdominal organs This would explain why in a few

experiments in section D of Chapter IV sympathetic block was found to increase the vagal effect on the jejunum as well though never more than to result in jejunal and ileal responses of approximately equal magnitude

The sympathetic inhibitory nerve fibres to the small intestine have generally been considered to exert their effect by a direct action on the smooth muscle cells (see e g YOUMANS 1952 BROWN DAVIES and GILLESPIE 1958) The present study however suggests that the sympathetic inhibitory fibres exert their effect at the intestinal ganglionic level Otherwise it would be difficult to explain why the tonic activity of the sympathetic inhibitory fibres to the ileum did not depress the effect of acetylcholine when it was given by way of the blood stream so that it also reached the smooth muscle cells (see Chapter IV section A and Fig 6) while it markedly suppressed the effect of acetylcholine when it was released locally at the preganglionic vagal nerve endings Recent histochemical studies of the adrenergic nerve terminals in rats and cats (NORBERG 1964) have also presented strong evidence that most adrenergic nerves to the intestine terminate in the enteric plexuses The smooth muscle layers receive very little adrenergic innervation and what they do receive probably belongs to the blood vessels It is suggested that the neurogenic adrenergic inhibitory effects observed in the present experiments were exerted only on the postganglionic parasympathetic ganglion cells and not on the smooth muscles Adrenergic inhibition of the smooth muscles directly would normally then occur only by way of the blood stream i e by way of catechol amine release from the adrenal medulla

The nervous control of the intestinal vascular bed is exercised by tonically active vasoconstrictor fibres in the splanchnic nerves These fibres induce a diversion of the intestinal blood flow from the intestinal mucosa to other parts of the intestine and constrict the capacitance vessels as well The total intestinal blood flow through the intestine however in the steady state is relatively little restricted by the constrictor fibre influence at least when compared with tissues such as skeletal muscle or skin (FOLKOW LEWIS LUNDGREN MELLANDER and WALLENTIN 1964 a b) This response pattern seems to be of functional significance e g in the defence reaction by which mobilization of blood from the intestinal capacitance vessels can be brought about and by enforcing an interruption of the digestive activity in the mucosa by depriving it of its blood supply (COBBOLD FOLKOW LUNDGREN and WALLENTIN 1964) In addition to the vasoconstrictor fibres the intestinal blood vessels have sometimes been assumed to be controlled by vagal vasodilator fibres as well However if such fibres really exist they are in any event not engaged in the baro and chemoreceptor control of the blood flow (CELANDER and FOLKOW 1951) and their functional significance may then perhaps be

linked to the intestinal function *per se*. As previously mentioned, however, it was impossible in the present experiments to induce any vasodilator responses in the intestine, whether by vagal stimulation in the neck, at the intrathoracic or the subdiaphragmatic level. Parasympathetic denervation of the intestine did not induce any discernible effects on intestinal blood flow which is in accordance with the results of FOLKOW, LUNDGREN and WALLENTIN (1963). Thus it seems justified to conclude that the vasoconstrictor fibres involved in e.g. the defence alarm reaction and the homeostatic blood pressure control (see ÖBERG 1964) constitute the only extrinsic innervation of the intestinal blood vessels.

Great precaution must be taken in assessing the functional significance of the present experiments, particularly concerning the integration of the parasympathetic and sympathetic influences of the gastrointestinal motor activity. In the intact organism the truly complex interaction of such mechanisms as effector automaticity, local spinal and supraspinal reflex arcs, blood borne and local chemical influences must be considered simultaneously. Nevertheless it seems justified to discuss some aspects of the functional significance of the present findings although they are to some extent conjectural.

From studies of the rate at which a contrast medium or a telemetring capsule traverses the small intestine, it is well known that the transit time is more rapid in the upper parts of the intestine than in the lower parts (see CONNELL, 1961). From a functional point of view this seems to be a suitable arrangement since the upper part of the intestine receives the gastric contents while a slower transport further down would be appropriate for digestion and absorption. This will be particularly true for substances such as fat and protein which are absorbed more slowly while it will be of lesser significance for substances such as glucose, iron and water soluble vitamins which are relatively more rapidly absorbed and where the absorption is usually complete before the ileum is reached. The difference in transit time may possibly be explained at least in part by the present findings. It seems to be generally accepted that the extrinsic nerves are concerned with the regulation rather than the initiation of the intestinal motor activity. These nerves serve to increase or decrease the motor activity induced by the different local mechanisms (see e.g. YOUMANS 1952). Thus the more rapid passage of food through the jejunum might depend on the relative lack of sympathetic inhibitor fibres to this part of the intestine while the longer transit time through lower parts might be explained by the presence of such fibres which would tend to suppress the activity of the intrinsic nerve plexus and the excitatory influence of the vagal nerve fibres. It may be argued that essentially the same effects would be obtained if the activity of the vagal fibres were more intense in the

upper parts of small intestine However a clear differentiation in discharge rate would then be needed since the present study indicates that the entire small intestine is equally innervated by vagal fibres Further a reduction in vagal excitatory discharge to lower parts of the small intestine alone would probably not induce a sufficient diminution of the intestinal motor activity in these sections as it is well known that the propulsive motility depends on strictly local intrinsic reflex arcs activated by the intestinal content (BÜLBRING *et al* 1958 HUKUHARA *et al* 1958) It seems therefore more reasonable to assume that an adequate reduction and control of the ileal motor activity is mainly dependent upon the relative abundance of specific inhibitory fibres distributed to this intestinal section and that these inhibitory fibres exert their action directly on the local neurones which are responsible for the intrinsic reflexes

Furthermore there does not seem to be any great difference in the absorptive capacity of jejunum and ileum for most substances Therefore it is possible that the above mentioned difference in extrinsic nervous control and thus in transit time between the upper and lower parts of the small intestine can be the explanations as to why more extensive resections of the distal small intestine cause a more significant degree of malabsorption than a corresponding resection of the proximal part (see e g Booth 1961)

In addition to the above mentioned innervation the small intestine is also controlled by strictly spinal reflexes in the form of the so called intestino intestinal inhibitory reflex which causes an immediate inhibition of tone and rhythmicity throughout virtually the whole small intestine when e g one intestinal segment is distended (see Chapter V) Similar responses can also be induced by peritoneal stimulation It seems less likely that these reflexes which for their activation necessitate fairly intense distentions are involved in the normal control of the intestinal motor activity concerned with digestion and absorption It seems more logical to suppose that they are activated mainly in pathological situations as protective reflexes during for instance peritonitis and intestinal obstruction

Finally it should be recalled that adrenomedullary hormones play an important role in the regulation exerting their inhibitory action also on the smooth muscles directly Thus the release of adrenaline seems to be responsible for the type of generalized inhibitions of intestinal motor activity seen with centrally induced excitations of the sympatho-adrenal system (see KOCK 1959)

However much remains to be done before the complex integration of the parasympathetic and sympathetic extrinsic influence on the intestine is fully understood In particular the organization of the sympathetic influence appears to be far more complex than earlier assumed

Summary and conclusions

The vagal influence on the jejunal and ileal motility and blood flow were studied by means of graded vagal stimulations at the cervical intrathoracic and subdiaphragmatic levels in urethane chloralose anaesthetized cats The responses in the two parts of the small intestine to graded stimulations of the splanchnic nerves were also investigated as were the responses to intravenously administered acetylcholine and adrenaline The jejunal and ileal motor activity were recorded continuously by registering changes in the intraluminal volume at a relatively constant transmural pressure and the blood flow through the two intestinal loops was measured as the venous outflow

On the basis of the results obtained in the present investigation the following conclusions seem to be justified

1 The jejunal and ileal sections of the small intestine are supplied with vagal excitatory fibres to approximately the same extent However while vagal stimulation induced motor responses in the jejunum in all experiments the responses of the ileum were insignificant or absent in about half of the experiments

2 This difference was shown to be due to the action of inhibitory fibres in the splanchnic nerves distributed mainly to the ileum Such an inhibitory neurogenic action could completely suppress the vagal excitatory influence on the ileum These sympathetic inhibitory fibres seem to exert their action at the ganglionic level in the ileum

3 The vagal nerves at the neck level do not contain any inhibitory fibres to the small intestine

4 The physiological range of impulse discharge of the vagal motor fibres to the small intestine seems to be between zero and 8—10 imp /sec in agreement with other autonomic neuroeffector systems

5 Depending upon the stimulus strength intrathoracic and subdiaphragmatic vagal stimulation induced either excitatory or inhibitory motor responses in jejunum and ileum The inhibitory responses were caused by activation of high threshold fibres and were accompanied by a decrease in the [illegible] blood flow and an increase in the blood pressure The nature of these [illegible]

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From the Department of Histology Karolinska Institutet and King Gustaf V Research Institute Stockholm Sweden

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Contents

To my family

Introduction

Since it was recognized that the FFA[1] in plasma constitute the main transport form of fat from the TG stores in adipose tissue to various parts of the body (GORDON and CHERKES 1956), there has developed an ever increasing interest in the problems concerning lipid mobilization and its consequences Fat is now known to be an important source of energy for most tissues except nervous tissue (for reviews see e g FREDRICKSON and GORDON 1958 FRITZ 1961) The heart depends on oxidation of fatty acids for the major part of its energy expenditure under fasting and resting conditions (cf GORDON and CHERKES 1956) During sustained work fat is also the main fuel for skeletal muscle (HAVEL *et al* 1963) The mobilization of FFA from adipose tissue is markedly enhanced in fasting (GORDON and CHERKES 1956) and during various kinds of stress, e g exposure to cold (GILGEN *et al* 1962) In such situations the enhancement of FFA mobilization has been interpreted as an adjustment of the organism to increased energy demands Accordingly the adipose tissue would be pictured as a tank from which fuel streams as the throttle is opened

However the transport of FFA from adipose tissue is but one step in a rather complex circuit of fatty acids (cf DOLE 1964) It appears that fatty acids may be esterified and released several times before being utilized as fuel For instance part of the FFA taken up by the liver will be recirculated in the blood as TG coupled to lipoproteins (cf CARLSON BOBERG and HOGSTEDT 1965) From these the fatty acids may again be detached and reenter adipose tissue cells Or i a in the myocardium lipoprotein fatty acids may be taken up together with FFA coming directly from the adipose tissue (GOUSIOS *et al* 1963)

In spite of the fact that there is an increased mobilization of FFA in situations when apparently, more fuel is needed all of them are not oxidized immediately on entering tissue cells Instead they may be esterified once more and mixed with the intracellular fat pools that appear to serve as the real reservoir of fuel for immediate use As this reservoir may be repleted—and very probably is—also in the

Abbreviations FFA = free fatty acids TG = triglycerides NA = noradrenaline SDH = succinic dehydrogenase

fed and unstressed individual one may in fact ask whether the enhanced release of FFA from adipose tissue is the result rather than the purpose of an overall reaction of the organism to stress or fasting At any rate it is remarkable that vast amounts of fuel mobilized in an emergency situation should not be wanted for immediate utilization The intracellular fat pools may play a much more important role than was earlier recognized

Most work in this field has been done by biochemical and physiological methods The main interest has been focused on factors governing the release of FFA from adipose tissue and the uptake and handling of them in various tissues Among the so-called adipokinetic (fat mobilizing) agents tested NA which is also the main sympathetic transmitter has been found to be effective both in vivo and in vitro in a number of species including man dog and rat (cf RUDMAN 1963) As sympathetic blockade may prevent the rise of FFA levels in plasma observed in fasting (HAVEL and GOLDFIEN 1959) cold exposure (GILGEN *et al* 1962) or after trauma (CARLSON and LILJEDAHL 1963a) the release from adipose tissue is believed to be at least in the species mentioned under the influence of the sympathetic nervous system As regards the uptake of FFA in tissues the plasma flux has been stated as the major determining factor (for references see Paper IV)

The precise relation of sympathetic nerves to adipose tissue cells cannot however be determined by biochemical or physiological methods nor can the reaction of individual tissue cells to increased flux of plasma FFA be evaluated The rapid development in recent years of sensitive and accurate morphological and histochemical methods provides some of the tools required for examining in more detail the very scene of action

The work presented in the following chapters was started with the intention of tying together biochemical physiological and morphological aspects of lipid mobilization and of presenting a morphological basis for the interpretation of biochemical and physiological findings

Part I

CHAPTER I

General Features of Excessive FFA Mobilization

If one adheres to the picture of the adipose tissue as a tank from which the fuel flow is regulated according to demand any mobilization of FFA that causes fatty acids to be deposited in the cells instead of being immediately oxidized is excessive However, in view of the normal occurrence of fat deposits in many cells the term excessive FFA mobilization should be restricted to denote such an enhancement of the physiologic process that deposition of fat intracellularly becomes a dominating feature

A marked increase in the amount of TG and stainable fat in various organs notably the liver (fatty liver), has been observed after administration of catecholamines (McKAY 1937 FEIGELSON *et al* 1961) Early evidence points to mobilization of depot fat as a possible cause of this phenomenon although the connection with enhanced release of FFA resulting in raised plasma levels, was not established until recently (FEIGELSON *et al* 1961)

Fatty liver is not an uncommon finding at post mortem examinations especially in post traumatic and other stressful conditions After trauma such as fractures burns and operative procedures there is a significant increase in the plasma levels of FFA as well as in the urinary output of catecholamines (FRANKSSON *et al* 1954, WADSTROM 1961 BIRKE *et al* 1957, 1964) In the first paper of a series on lipid metabolism and trauma CARLSON and LILJEDAHL (1963a) reported that experimental trauma in the dog induced a rise in plasma levels of FFA together with increased TG content in the liver and appearance of stainable fat in liver and myocardium These changes could be prevented by previous administration of guanethidine a peripherally acting sympathetic inhibitor The effect of trauma was similar to that of NA infusion although the latter produced more extensive changes

The observation that NA infused dogs also showed symptoms similar to those sometimes observed in post traumatic conditions —fever, tachycardia, increased respiratory frequency and even dyspnea—aroused the interest in excessive FFA mobilization as a possible clue to certain complications in patients subjected to severe trauma The present studies were initiated as part of an extended analysis of the events following enhanced release of FFA from adipose tissue It was thought that the results might be of value, not only for the understanding of traumatic complications and their pathophysiology, but also for our knowledge of lipid metabolism in general

Preliminary investigation of tissue changes after excessive FFA mobilization (Paper I)

This paper presents a preliminary survey of the distribution of intracellular fat droplets after excessive FFA mobilization, induced by continuous NA infusion in the dog

Small sudanophilic droplets appeared after 4 hours of NA infusion (0 5—1 0 μg per kg body weight and minute, clinical dose in man up to 0 2 μg) in liver myocardium lung diaphragm and kidney which were the organs taken out for histological examination In the liver the distribution of droplets in the lobules seemed to be related to the direction of the blood flow as most sudanophilic droplets were seen in periportal areas from which the blood flows towards the central veins The myocardial fibers were homogeneously affected In lung diaphragm and kidney however, the distribution was heterogeneous with droplets confined to alveolar macrophages and bronchiolar epithelium, to some fibers only in the diaphragm, and to the straight part of proximal tubules As the infusion was prolonged the changes became more accentuated as at 24 hours the liver cells were filled with droplets throughout and abundant droplets were seen also in the other organs However, the heterogeneous pattern in lung diaphragm and kidney prevailed

As the biochemical analysis showed a marked increase in the TG content of the liver the sudanophilic droplets were interpreted as TG deposits formed by esterification of FFA that had been taken up from plasma However since the picture resembled that obtained in cell damage resulting from e g anoxia or poisoning supplementary evidence had to be gathered in order to make certain

whether the observed changes were due to an overload with FFA alone Therefore some specimens (kidney, myocardium and skeletal muscle) were prepared for electron microscopy (together with A B MAUNSBACH) No conspicuous signs of cell damage were noted

The only indication of tissue damage was found in the lung specimens from animals infused for 24 hours There were small atelectases and sometimes leucocytic infiltration Characteristically clusters of fat laden cells were seen in the atelectatic areas and on the borders of still open alveoles In the discussion of this paper the hypothesis was forwarded that the alveolar tissue—except for the fat laden cells—could not manage to esterify the excess FFA which might therefore have some toxic effect At the time of these preliminary experiments it was still generally assumed that the flux of FFA from plasma into cells was mostly an obligate process

However the heterogeneous distribution of fat droplets in organs that displayed heterogeneous staining reactions also with other methods e g for esterase and glycogen tended to contradict this assumption Abundance of fat droplets e g in the kidney and lung was fairly regularly related to strong nonspecific esterase activity This aroused the first—though yet tacit—suspicion that there might be a selective uptake of FFA

Conclusions from the preliminary investigation

The occurrence of sudanophilic droplets in the cells of various tissues after enhancement of FFA mobilization by infusion of NA is proposed to be due mainly to increased deposition of fatty acids taken up and esterified

The droplets not only in the liver but in other organs as well in all probability represent intracellular TG stores

The cells have not been visibly damaged by the excessive FFA mobilization except possibly in the alveolar tissue of the lung Thus increased flux of FFA into cells should be borne in mind as a possible explanation of fatty changes which are still *often interpreted* as due to unspecified toxic damage

There is a remarkable correlation between the distribution of fat droplets and histochemical homo- or heterogeneity

Scope of the Extended Investigation

Two fundamental problems among those presenting themselves in the preliminary investigation appeared especially suitable for further penetration, namely, the distribution of sympathetic nerves in adipose tissue and the relation between histochemical characteristics of cells and their uptake of FFA Thanks to the fluorescence method elaborated by HILLARP and coworkers (see Chapter III), it is now possible directly to demonstrate the localization of biogenic monoamines in tissues and thus the extension of the sympathetic nervous system The development of the cryostat technique makes it possible to apply a wide range of histochemical staining reactions, to a great extent without previous fixation, to one single piece of tissue and even to one and the same cell in serial sections The combination of freeze sectioning and autoradiography as outlined by ULLBERG (see Chapter III) provides excellent means of localizing labeled compounds among them fatty acids without exposing the tissue to water or organic solvents

During the work it became increasingly apparent that skeletal muscle holds a central position both in fatty acid metabolism and in the problems concerning it Skeletal muscle comprises nearly half of the body mass and is one of the main consumers of fatty acids It is metabolically heterogeneous consisting of fibers that depend variedly on fat and carbohydrates for generating energy (for references see Papers IV, VI and VII) Muscle is easily available for taking specimens and the length of the fibers permits practically unlimited series of staining reactions to be made on the same cell Therefore some morphological and histochemical aspects of the metabolism of fatty acids in skeletal muscle were finally studied

The scope of the extended investigation may be outlined as follows (Roman numerals denote the paper[s] dealing with the respective problems)

1 To examine the distribution of adrenergic sympathetic nerve fibers in adipose tissue in order to clarify the spatial relationship

between the sympathetic nervous system and adipose tissue cells (II)

2 To evaluate the importance of the local adrenergic innervation in adipose tissue by comparing it in two species with different responsiveness to NA as an adipokinetic agent (II III)

3 To study the correlation between plasma lipid changes and the appearance of intracellular fat droplets (IV)

4 To study the ultrastructure of intracellular lipid deposition and to compare electron microscopical and histochemical findings (V)

5 To study the depletion of intracellular lipid deposits (IV V)

6 To present some morphological and histochemical aspects of a metabolically heterogeneous tissue (skeletal muscle) with respect to

a) development of heterogeneity during fetal life (VI)

b) relation between histochemical parameters and lipid deposition (IV V) and

c) relation between the amount of stainable fat and the uptake of FFA in different fibers (VII)

Material and Methods

Material

The choice of experimental material was generally determined by the extent to which it had been used in biochemical and physiological work on the same problems The following material was investigated

White mammalian adipose tissue (epididymal or parametrial fat) from rat and rabbit
White avian adipose tissue (pericloacal) from domestic fowl
Brown adipose tissue (interscapular bodies) from rat and hedgehog
Mixed brown and white adipose tissue (cervical) from guinea pig
Liver myocardium diaphragm gracilis muscle lung and kidney from dog
Trunk musculature and lung from fetal and newborn mice,
Pectoralis major and minor muscles from pigeon

Care was always taken to get as fresh material as possible preferably from the living animal At the biopsies of liver and muscle in the dog experiments the vitality of the tissue was always ascertained by inspection and each biopsy performed at an appropriate distance from the preceding one At autopsy (dog experiments) sampling was generally completed within 2 to 3 minutes the first specimens (muscle and liver) being removed from the still living anesthetized animal which was then killed as the heart was taken out

Methods

Histochemical demonstration of NA (fluorescence method of HILLARP and co workers extensively reviewed by DAHLSTRÖM and FUXE 1964 NORBERG and HAMBERGER 1964)

Adipose tissue specimens were frozen in propane cooled with liquid nitrogen and frozen-dried Treatment with formaldehyde gas (relative humidity 30—50 per cent, HAMBERGER *et al* 1965) for 1 hour at +80°C was followed by embedding in paraffin *in vacuo* sectioning at 10 μ and

mounting in Entellan[R] (Merck, Darmstadt, Germany). The sections were examined in a Zeiss monocular microscope (Osram HBO 200 u.v. lamp); 3 mm Schott BG 12 and 1 mm KG 1 filters for activation and a Zeiss 50 filter as stop filter in the tube).

Primary catecholamines are condensed with formaldehyde to form 1,2,3,4-tetrahydroisoquinolines, which in a second reaction, catalyzed by protein present in the section, are transformed into 3,4-dihydroisoquinolines with an intensely green to yellow green fluorescence. The reactions require the presence of water. As, however, both catecholamines and their reaction products are soluble in water, there is a range of optimum humidity at which there may be good fluorescence with but minimal diffusion. Adipose tissue presents special problems owing to the difficulty in drying it completely. Therefore relatively dry formaldehyde was used.

In its present form, the method is specific for primary catecholamines, but no for NA in particular. However, the only primary catecholamine present in peripheral sympathetic nerves of the species investigated would be NA. The visualization of nerve terminals may be improved either by pretreatment of the animal with a monoamine oxidase inhibitor, or by injection of NA, or incubation of the specimen with NA (HAMBERGER and MASUOKA 1965). The last two modifications are based on the uptake of NA by the terminal part of the adrenergic neuron.

Histochemical methods for lipids, carbohydrates (glycogen) and enzymes

The two main sources of energy in the body are fatty acids and carbohydrates, stored in the cytoplasm mainly in the form of TG and glycogen. Since there is a reciprocal relationship between them as regards utilization, a survey of the distribution and amount of glycogen in tissues is important in the study of lipid metabolism. However, staining of fat and glycogen only traps the specimen in a certain metabolic situation; some other histochemical methods are therefore necessary to demonstrate the metabolic pattern of each cell, whether it is directed towards utilization of fat or carbohydrates, or both.

As the greatest changes during excessive FFA mobilization occur in the TG fraction as shown by biochemical analysis, the stainings for lipids were limited to such demonstrating the hydrophobic neutral lipids. Three enzymes were chosen to represent the general metabolic pattern of cells, namely, succinic dehydrogenase (SDH), which takes part in the oxidation of fatty acids, α-naphthyl acetate esterase, or nonspecific esterase, which is actually a mixture of enzymes of which some may be active in the esterification of fatty acids, and phosphorylase, which is stated to be the glycogenolytic enzyme. No attempt was made to demonstrate e.g. lipase or other enzymes more specifically involved in lipid

metabolism partly because e g the lipase methods are still very unsatisfactory Moreover the enzyme reactions studied here have been extensively investigated with respect to their distribution and intensity i a in the different types of muscle fibers

In skeletal muscle peroxidase activity interpreted as due to myoglobin was also studied

Fixation and sectioning techniques For general survey of lipid distribution pieces from the specimens taken in the dog experiments (Papers I and IV) were fixed in 10 per cent ice-cold neutral formalin and then freeze sectioned at 10 and 20 μ on an MSE sledge microtome with CO_2 freezing attachment

Freezing techniques offer essential advantages in the preservation also of carbohydrates and enzymes and were therefore applied in the preparation of the histochemical series In the earlier experiments freeze drying and embedding in polyethylene glycol were used but were later replaced by the more rapid and convenient sectioning of frozen material in a cryostat (W Dittes Heidelberg Germany)

The cryostat technique permits sectioning of unfixed as well as fixed material at constant temperature (usually —20 C) thus avoiding artifacts caused by repeated freezing and thawing as in the ordinary freeze sectioning technique The section thickness can be kept as low as 2 μ however in the present investigation the sections were made at 7—15 μ Serial sections were made throughout (see schedule p 19) The unfixed material was rapidly frozen in isopentane cooled with liquid nitrogen or in liquid nitrogen only no significant difference was noted between the results obtained with either method Fixation was performed with formol sucrose at +4 C (4 per cent formaldehyde in 7 5 per cent sucrose buffered with 0 067 M phosphate buffer according to HOLT and HICKS 1961) for 24 hours The specimens were then kept in 7 5 per cent sucrose with 2 per cent gum acacia at the same temperature up to sectioning in the cryostat

All specimens for histochemical examination were dissected so that at least one dimension was below 3 mm in order to ensure uniform freezing and fixation

Lipid staining (Sudan III—IV Sudan Black B as modified after MEIER 1959) The formalin fixed freeze sectioned preparations were stained with a mixture of Sudan III and IV in 70 per cent ethanol and acetone 1 1 with Ehrlichs or Harris hematoxylin as counterstain (cf BARKA and ANDERSON 1963) As this stain however gave very weak contrast in frozen dried or cryostat sectioned preparations these were instead stained with Sudan Black B supersaturated in 70 per cent methanol as

modified after MEIER (1959) (staining 5 minutes subsequent rinses in 70 per cent methanol 2—3 minutes)

Sudan III—IV is an excellent stain for demonstrating i a TG stores as it stains practically only nonpolar lipids Sudan Black B was used in a solvent that has been found to provide optimal adsorption conditions at the solvent lipid interface (this is actually the crucial step in all staining of nonpolar lipids) However the stain is not available in pure form but consists of several components some with affinity for nonlipid material Moreover Sudan Black B stains phospholipids in mitochondria and cytomembranes as well and is thus less specific although more sensitive than Sudan III—IV

Glycogen staining (PAS reaction as modified by MOWRY *et al* 1952) The periodic acid-Schiff reaction (PAS) was performed in 70 per cent ethanolic solutions as recommended by MOWRY *et al* (1952) to diminish dissolution of low molecular polysaccharides The sections were preincubated in 80 per cent ethanol (test sections) diastase (Merck Darmstadt Germany) 1 per cent in physiological saline (control sections) or in saline only (control of diastase effect) for 1 hour at room temperature (GRAUMANN and CLAUSS 1959) Diastase-digestible water resistant material was interpreted as glycogen The reaction product is magenta colored

By this reaction, 1 2 glycols are oxidized to dialdehydes which are Schiff positive For all practical purposes most of the 1 2 glycols in tissues are present in polysaccharides Of the two glycogen fractions (lyoglycogen and desmoglycogen) only the lyoglycogen appears to be PAS positive whereas the desmoglycogen remains unstained probably due to blocking glycogen protein bonds (KUGLER and WILKINSON 1959 1960) As however lyoglycogen is believed to be the mobile fraction PAS staining should be suitable for demonstrating within certain limits differences in the extent of glycogen deposition or utilization at different stages of FFA mobilization experiments

Succinic dehydrogenase (SDH) (PEARSON 1958 PEARSE 1960) Fresh frozen cryostat sections were incubated for 30 or 45 minutes at room temperature in a medium containing 10 per cent 0 2 M sodium succinate 40 per cent nitro blue tetrazolium (Nitro-BT) 20 per cent N N -dimethyl formamide 20 per cent 0 1 M phosphate buffer pH 7 6 and 10 per cent redistilled water Controls were made by omitting sodium succinate The reaction product is a blue black microcrystalline formazan

Nitro-BT was used because it is stated to be insoluble in lipids N,N dimethyl formamide was added to enhance the otherwise very slow diffusion of Nitro-BT into the section in order to provide sufficient amounts for visualizing the enzyme reaction (PEARSON 1958) The localization of the formazan has been claimed to be intramitochondrial corresponding fairly

well with that of the enzyme (SEDAR and ROSA 1961 YAEGER 1961) The validity of this claim will be further discussed in Chapter VIII

Nonspecific esterase (α naphthyl acetate esterase) (PEARSE 1960) Frozen-dried or fresh frozen cryostat sectioned material postfixed for 2 hours at 37°C over paraformaldehyde was used together with formol sucrose fixed cryostat sectioned Incubation was performed for 5 minutes at room temperature in a medium containing 0 1 per cent α naphthyl acetate and 0 1 per cent Fast Blue B in 0 1 M phosphate buffer pH 7 4 (PEARSE 1960) Controls were made by omitting α naphthyl acetate The reaction product is a granular or diffuse brown or brownish red precipitate

Many enzymes split acetate esters Due to the possible inhibition of some of them with formalin or to dissolution of others in paraformaldehyde fixed sections the results obtained with the two methods for pretreatment are not entirely comparable (cf BARKA and ANDERSON 1963)

Phosphorylase (TAKEUCHI and KURIAKI 1955, modified by ERANKO and PALKAMA 1961) Fresh frozen cryostat sections were incubated for 1 hour at room temperature in a medium containing 50 mg glucose 1 phosphate 5 mg adenosine 5 phosphate 1 mg glycogen 1—2 I U insulin (Insulin Vitrum, Stockholm, Sweden) 90 mg sodium fluoride 450 mg polyvinyl pyrrolidone (PVP) and 1 ml absolute alcohol in 5 0 ml 0 1 M acetate buffer pH 5 9 The reaction product (amylose) was visualized by the iodine reaction with Gram s solution in 0 32 M sucrose after drying the sections for 1 hour at 37 C The sites of phosphorylase activity are indicated by a blue or bluish brown precipitate

In the presence of insulin and adenosine 5 phosphate phosphorylase adds *glucose to the chains of the glycogen primer Sodium fluoride is present to* inhibit glycolytic interference PVP (ERANKO and PALKAMA 1961) increases the viscosity of the medium and prevents diffusion of the reaction products Previously formed glycogen is dissolved and gives no stain The iodine starch reaction fades in a few days but can be redeveloped by repeated incubation with Gram s solution as described (author s modification)

Peroxidase (myoglobin) (DREWS and ENGEL 1961) Formol sucrose fixed cryostat sections were pretreated with benzene and then incubated for 8 minutes in a medium containing 9 ml of a 0 5 per cent solution of benzidine hydrochloride in saline 1 ml of a saturated ammonium chloride solution and 1 drop of a 3 per cent solution of hydrogen peroxide The reaction product is a crystalline blue precipitate (quinhydrone) which after few hours turns to a brown quinoneimine with considerably less contrast The sections were therefore immediately photographed

The original description gives good evidence that the peroxidase visualized in muscle fibers is myoglobin Formol sucrose fixation appears to have im proved the clarity of the reaction (originally formalin was recommended)

Serial sectioning schedule (skeletal muscle + mouse fetuses)

Fresh frozen	*Formol sucrose*
PAS (test section)	Myoglobin
Sudan Black B	PAS (test section)
PAS (water control)	Sudan Black B
Esterase	PAS (water control)
PAS (diastase control)	Esterase
SDH	PAS (diastase control)
Phosphorylase	

Each series was repeated once Section thickness 7—8 μ (earlier series) later 15 μ

Autoradiography (ULLBERG 1954)

For autoradiography of intravenously injected albumin bound 1-C^{14} palmitate in pigeon pectoralis muscles freeze-sectioning with adhesive tape as section vehicle was applied after freezing the specimens in liquid nitrogen 3 6 or 15 minutes after the injection Apposition autoradiography was performed with Gevaert D 7 Structurix for 3 to 15 weeks

Autoradiography does not actually demonstrate the localization of the administered substance but that of the radioactive isotope For reasons given in Chapter IX most of the isotope was considered to be contained within esterified fatty acids in the sections

Electron microscopy

For electron microscopy specimens from the same pieces of tissue as those used for light microscopy were taken and fixed osmium tetroxide solutions and embedded in Epon 812 as described in Paper V Exami nation of the sections cut with an LKB Ultrotome or on a Porter Blum ultramicrotome was performed in an RCA 3A electron microscope or a Siemens Elmiskop I

Biochemical methods

Titration of plasma fatty acids was made according to DOLE (1956) and determination of tissue and plasma TG as described by CARLSON (1960 1963)

Notes on Terminology

Adrenergic nerve terminals The terminal parts of the sympathetic adrenergic neurons form an intricate network in the tissues innervated the so-called "terminal plexus (cf NORBERG and HAMBERGER 1964) On entering this plexus the axons assume the appearance of varicose threads with an extremely high NA content about ■ hundred to a thousand times that of cell bodies or axons Release of NA is considered to take place all along the varicose threads Consequently the terminal plexus as well as the free terminals are to be regarded as the adrenergic innervation apparatus Terms like 'sympathetic nerve endings" are inadequate and should be rejected

Alveolar wall cells In Paper I the fat laden cells in the lung were referred to as "alveolar macrophages" However it has been suggested that some of the cells in the alveoli elaborate a surface-active lining film to secure an even inflation of the lung (cf PATTLE 1965) There is now evidence that this substance may be secreted by a special type of cuboidal cell in the alveolar wall. The term alveolar wall cells was adopted after NAGAISHI *et al* (1964) who presented an electron microscopic survey of the alveolar structure in several species

FFA uptake vs extraction The terms uptake", 'removal and "extraction have been rather indiscriminately used in the literature FFA uptake will be used here to denote an unspecified process by which FFA leave plasma or an incubation medium to appear inside the cells The term FFA extraction" expresses the contention that not only permeability but also active transport determines the extent to which FFA enter the cytoplasm

Lipid deposition There are many terms to denote appearance of stainable fat in the cytoplasm. Common, though vague ones, are e g. "fatty changes" "fatty infiltration" and "fatty metamorphosis "Lipophanerosis" means that fat already present in invisible form is converted to cytoplasmic fat droplets In the present material the

stainable fat appearing in various tissue cells is as stated before in all probability derived mainly from mobilized FFA Therefore the term lipid deposition was eventually adopted Evidently this term may also be used of similar changes due to increased lipid synthesis in the cells

Lipolysis vs FFA release Lipolysis means splitting off of fatty acids from the glycerol moiety in glycerides thus making them available i a for oxidation In the white adipose tissue however only a minor part of the hydrolyzed fatty acids are oxidized They may, instead be reesterified with α glycerophosphate derived from glycolysis If the rate of lipolysis exceeds that of reesterification in the white adipose tissue the excess FFA will leave the cells and appear in plasma or in an incubation medium This is denoted as FFA release

Red white and intermediate skeletal muscle fibers These terms actually refer to the myoglobin content of each fiber type but are extensively used also by authors who have in fact never tested it However the three types may be identified also by other histochemical criteria such as e g SDH content which is higher in red than in intermediate and white fibers

Part II

CHAPTER V

Noradrenaline and the Adipose Tissue

There are two types of adipose tissue in the mammalian body white and brown White adipose tissue (commonly denoted as "fat") (subcutaneous omental, epididymal etc) is composed of large spherical cells with a thin cytoplasmic layer surrounding a single fat droplet ("unilocular" adipose tissue) this type of adipose tissue constitutes the principal storage site of TG in the body Brown adipose tissue is found i.a. as the interscapular fat bodies in most newborn mammals including man, and in newborn and adult hibernators and rodents It consists of polyhedral cells with abundant small fat droplets in the cytoplasm ("multilocular" adipose tissue) which is rich in mitochondria and glycogen.

The function of brown adipose tissue has been much discussed it was formerly believed that it differs from that of the white adipose tissue only quantitatively but recent findings of its high oxygen consumption—ten times that of white adipose tissue—and of its marked growth in cold adapted animals support the concept that brown adipose tissue is an important site for oxidation of fatty acids presumably as a means of heat production this has also been verified recently by temperature recordings (cf. Dawkins and Hull 1964)

The fact that white adipose tissue passes through a multilocular stage during fetal development has caused some confusion as to the ontogenetic relation between the two types There is evidence however that they develop as separate entities (Wassermann 1926, Napolitano 1963) The multilocular cells in avian adipose tissue are stated to be quite unrelated to those of mammalian brown adipose tissue (Clara 1930) However there may be areas of mixed brown and white adipose tissue e.g. in the rabbit and guinea pig giving a superficial resemblance to avian fat.

Earlier concepts of adipose tissue innervation

The evidence of a direct influence of sympathetic nerves on adipose tissue cells is seemingly conclusive. Nerve-like structures in close proximity to their cell membranes have been repeatedly visualized with histological methods, such as methylene blue staining (DOGIEL 1898) or silver impregnation (BOEKE 1933, HAUSBERGER 1934), although, owing to the inherent capriciousness of the methods, contradictory results were reported (RASMUSSEN 1924, NORDMANN 1926). However, unmyelinated nerve fibers between brown adipose tissue cells were unequivocally demonstrated by electron microscopy (NAPOLITANO and FAWCETT 1958). Denervation appeared to prevent mobilization of fat from both white and brown adipose tissue (WERTHEIMER 1926, SIDMAN and FAWCETT 1954). Biochemical analysis revealed the presence of NA in both types, although the content in white adipose tissue was considerably lower, 0.04 μg per g wet tissue weight compared to 1.4 μg for brown adipose tissue in the rat (STOCK and WESTERMANN 1963). Stimulation of nerves to adipose tissue, lastly, provoked an increased release of FFA both in vivo and in vitro (CORRELL 1963, ORO *et al.* 1965).

However, none of these findings prove, in fact, that adipose tissue cells are innervated from the sympathetic nervous system. The morphological methods, if at all specific for nervous structures, do not identify adrenergic nerves. Denervation influences several processes, such as vasoregulation, and is a rather rough measure for specifically depriving adipose tissue cells of an assumed nervous supply. Biochemical analysis of whole tissue extracts does not localize NA, nor does nerve stimulation, which only causes NA to be released somewhere in the preparation. Lastly, there are animals which are quite unresponsive to the adipokinetic action of NA (cf. RUDMAN 1963).

It appeared that localization of NA was the salient point.

Examination of the distribution of adrenergic nerve terminals in adipose tissue (Paper II)

This paper presents the first fluorescence microscopic findings obtained with the histochemical fluorescence method for NA as applied to brown and white adipose tissue from rat, rabbit and domestic fowl.

CHAPTER V

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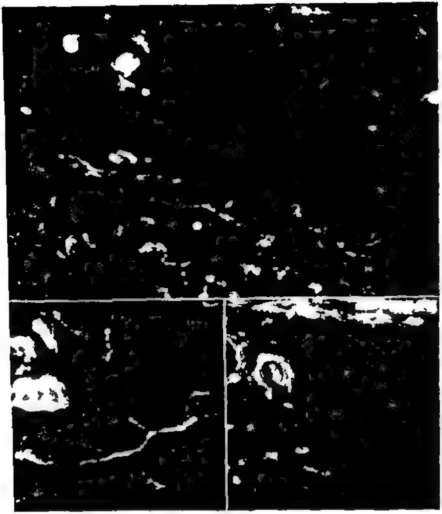


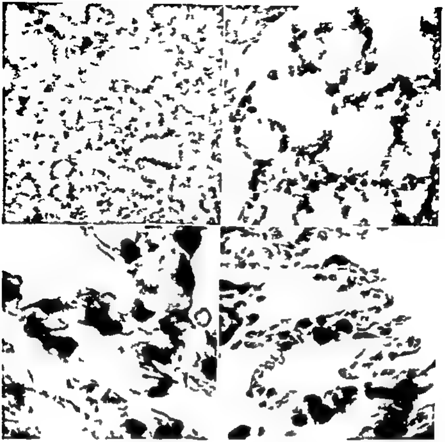
Plate 1 Adrenergic Nerves in Adipose Tissue

A White (upper half) and brown adipose tissue (lower half) Delicate terminals with varicosities in the brown adipose tissue none in the white 250 ×

B Brown adipose tissue Abundant fluorescence around vessels small terminals among adipose tissue cells 400 ×

C Fowl adipose tissue Fluorescent fibers in arterial wall 400 ×

(Chapter 1)



B

D

Plate 2 Alveolar Wall Cells

A Fetal mouse lung 17th gestation day weak esterase activity in cells in developing parenchyma 160 ×

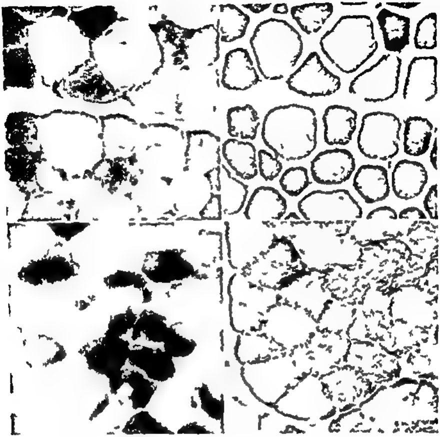
B Fetal mouse lung, 18th gestation day adult alveolar structure with strongly reacting alveolar wall cells 160 ×

C Dog lung with esterase positive alveolar wall cells 380 ×

D Dog lung NA infusion 24 hours Sudan III—IV Fat in alveolar wall cells. 380 ×

(Chapter VIII)

A



C

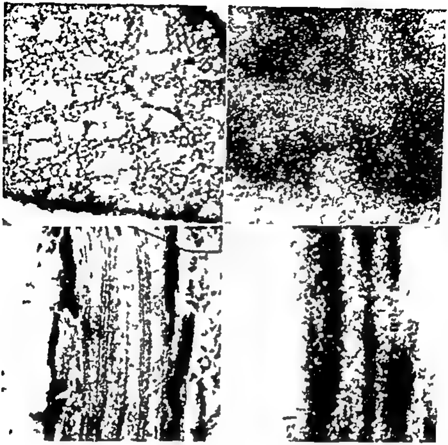
Plate 3 Lipid Deposition in Muscle

A Myoglobin B fat droplets (Sudan Black B) in formol sucrosefixed speci men from dog after 8 hours of NA infusion 160 ×

C Esterase D fat droplets (Sudan Black B) in fresh frozen specimen from dog after 8 hours of NA infusion 160 ×

Note that there are only two different intensities in the esterase stained section (C) as compared to the three seen in the others (A B and D)

(Chapter IX)



A B C D

Plate 4 The Selective FFA uptake

Sudan Black B stained sections (A C) and corresponding autoradiograms (B D) from pectoralis major of pigeon [illegible] minutes after intravenous injection of 1-C palmitate 40 ×

(Chapter IX)

at this laboratory from the experience gathered by examination of tissues from several species that an ordinary innervation of blood vessels may account for a NA content of up to 0 3 μg per g wet tissue weight, GILGEN *et al* (1962) had emphatically stated, however that the content of NA in white adipose tissue would be in excess of that required in vasoregulation Now this could still obtain if the local adrenergic innervation around the precapillary vessels were there to provide the NA necessary for stimulating FFA release

The validity of this assumption was tested in the following paper

The relation of responsiveness to NA as an adipokinetic agent and adrenergic innervation pattern of adipose tissue in rat and domestic fowl (Paper III)

This paper presents the first report on the effect of NA in vivo and in vitro in the domestic fowl compared to that in vitro in the rat In the in vitro tests the effects of adrenaline ACTH and glucagon in the two species were compared as well

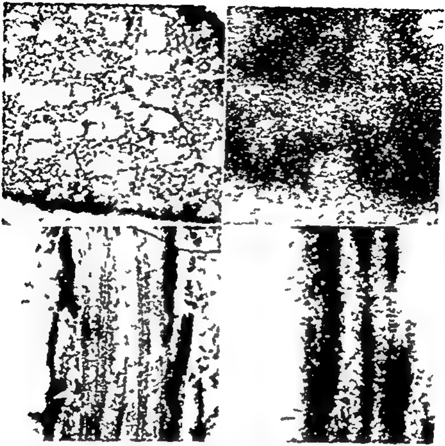
No effect of NA on FFA mobilization was noted in the domestic fowl although the innervation pattern and NA content in adipose tissue are similar to those in the NA responsive rat That the low release in the domestic fowl did not represent maximal stimulation was shown by the prompt response to glucagon which so far appeared to be the only adipokinetic agent common for the two species among the substances tested

Conclusions

In view of the present findings as well as of recent morphological and biochemical evidence there is no support for the concept of a direct innervation to white adipose tissue cells from the sympathetic nervous system In responsive species NA may be one of the humoral factors regulating FFA release

The present results emphasize the fundamental difference now increasingly recognized between brown and white adipose tissue as regards structure and function Brown adipose tissue is an important site of heat production most of the fatty acids obtained by lipolysis are retained within the cells and oxidized by the abundant mitochondria and the contribution to plasma FFA is insignificant (DAWKINS and HULL 1964) White adipose tissue is the principal source

A B



C

Plate 4 The Selective FFA uptake

Sudan Black B stained sections (A C) and corresponding autoradiograms (B D) from pectoralis major of pigeon 6 minutes after intravenous injection of 1-C^{14} palmitate 40 ×

(Chapter IX)

Part III

CHAPTER VI

Relation of Plasma Lipid Changes to Intracellular Lipid Deposition

Although the increased mobilization of FFA induced by NA infusion might be assumed to determine primarily the amount of intracellular lipid deposited in the dog experiments (Paper I) the possibility that plasma lipids other than FFA, e.g. TG in lipoproteins, had contributed to some extent could not be entirely ruled out. Triglyceridemia has been held responsible for lipid deposition in blood vessel walls; moreover, i.a. the myocardium may take up fatty acids from the lipoproteins in plasma (cf. Gousios *et al.* 1963). It was decided to elucidate this problem by stimulating FFA mobilization only during part of an infusion experiment and by studying the relation of the increase in FFA levels to subsequent changes in plasma and tissue lipids.

Role of FFA vs. other plasma lipids for intracellular lipid deposition (Paper IV)

This paper presents a follow up and extension of the investigation preliminarily reported in Paper I.

NA infusion was given continuously to dogs either during the whole of a 24 hour experiment or during the first 8 hours only. Increased FFA levels in plasma were found to be strictly related to the administration of NA and decreased promptly on terminating the infusion. In both groups the TG levels in plasma increased from about 12 hours onwards. The main increase was confined to very low density lipoproteins.

It was found that the only change in plasma lipids that contributed significantly to the intracellular lipid deposition was the increase in

plasma FFA. Since the increase of plasma TG in both groups was correlated to the increase in liver TG from [illegible] to 8 hours, it may be assumed that the developing triglyceridemia is rather a phenomenon parallel to intracellular lipid deposition, though with a certain time lag, and that the primary determinant of both is the enhanced mobilization of FFA. It is noteworthy that the amount of stainable fat in the myocardium, like in other organs, decreased after withdrawal of the NA infusion in spite of increasing plasma TG levels. That this increase is not due to a direct effect on the liver by NA per se is suggested by experiments with portal infusion of NA (FEIGELSON *et al.* 1961) or blocking of the FFA release induced by NA by means of nicotinic acid (CARLSON and LILJEDAHL 1963b), no significant changes in liver TG were induced.

The combined bio- and histochemical analysis of tissue lipids made possible an assessment as to the validity of either method for estimating the changes. It was found that the TG values corresponded fairly well with the observed amount of sudanophilic droplets in homogeneous organs, such as the liver. In myocardium and skeletal muscle, however, where the changes could be followed in detail histochemically, the biochemical analysis gave extremely varying results. This is mainly due to the fact that in these tissues there are interstitial fat cells, which are contained in the biopsy specimens in varying amounts. Roughly, one single fat cell with a diameter of about 100 μ would correspond to some five millions of intracellular fat droplets. In fact, histological examination proved to be the only reliable means of following the changes of intracellular lipid content in most organs studied.

Conclusions

Increased FFA levels are the primary determinant of intracellular lipid deposition during excessive mobilization of FFA.

Histological examination is essential for estimating the extent of lipid deposition in heterogeneous tissues.

Ultrastructure of Intracellular Lipid Deposition

The passage of fatty acids from plasma to cells is an as yet poorly elucidated process. Presumably the FFA are detached from plasma albumin and passed through the endothelium and basement membrane of capillaries to appear in the intercellular space from which they are eventually taken up by the cells. It is held that lipid may pass through an apparently intact cell membrane without any signs of pinocytotic activity (SJÖSTRAND 1963); however, no other information seems to be available concerning the transport of fatty acids during the passage now outlined.

In the cells the FFA are rapidly esterified and converted to phosphatidic acid which may then be further transformed into TG or phospholipids. There seem to be no reports specifically concerning the deposition of esterified fatty acids as seen in the electron microscope in tissues except adipose tissue (cf. NAPOLITANO 1963). It was therefore considered worthwhile to extend the electron microscopic investigation briefly reported on in Paper I.

Electron microscopic study of intracellular lipid deposition (Paper V)

This paper presents a comparative light and electron microscopic study of lipid deposition in kidney, myocardium and skeletal muscle of the NA infused dog. The specimens were taken from the series described in Papers I and IV.

The first ultrastructural sign of intracellular lipid deposition was found to be the appearance of structures referred to as "lipid bodies", as they corresponded closely to the sudanophilic droplets seen in the light microscopical preparations with respect to size, amount and distribution. They were rounded and contained an amorphous material with varying electron density, often partially dissolved, and lay free in the cytoplasm in the kidney preparations; in sections from skeletal muscle or myocardium they were invariably associated with mitochondria, although there was no obvious relation to the membranes of the sarcotubular system. No membrane structure was seen around the lipid bodies. The electron dense material in them was in

all probability TG, no explanation for the unusually low contrast or for the dissolution of this material can be offered, as the reactions of lipids to various steps in the preparation procedure are as yet very incompletely elucidated

The *findings corroborated the contention* in Paper I *that there* had occurred no obvious damage to the cell structure during the enhanced FFA mobilization The increase, not demonstrable histochemically, in the amount of glycogen like particles after 8 hours of NA infusion is noteworthy since it visualizes the decrease in glycogenolysis when excess fatty acids are available

Conclusions

The deposition of lipid in hydrophobic form as distinct droplets appears to occur in the free cytoplasm without any close relation to intracellular membrane systems

No information concerning the transport of FFA from plasma into cells can as yet be obtained with electron microscopic technique

Depletion of Intracellular Lipid Stores

If the experiments with NA infusion in dogs are held to represent an acceleration of the physiologic fatty acid transport to tissues there may be an enhancement of the physiologic processes for utilization of the fatty acids as well There are at least two ways conceivable for the depletion of intracellular lipid stores namely secretion of the fatty acids and oxidation The rising plasma TG levels suggest augmented secretion of fatty acids from the liver In recent years it has been suggested that the so-called alveolar wall cells of the lung may secrete considerable amounts of fatty acids in the lecithin-containing surface active alveolar lining film (NAGAISHI *et al* 1964, SCHAEFER *et al* 1964, for a review, see PATTLE 1965)

Oxidation of fatty acids is an important source of energy for most tissues as stated in the introduction It has been suggested that the rate of oxidation may be influenced by the amount of fatty acids available in free form in the cytoplasm (EATON and STEINBERG 1961) However according to present evidence this amount would probably not increase on enhancement of FFA mobilization since the fatty acids taken up will be esterified to a major extent It would be of considerable interest to try to find out whether the increased flux of FFA to cells might in some indirect way influence the oxidation rate by adding to the size of the intracellular lipid pools

Evaluation of some conceivable mechanisms for the depletion of intracellular lipid stores (Papers IV and V)

The amount of stainable fat decreased in all organs examined notably skeletal muscle after withdrawal of the NA infusion So did also the liver TG content, as judged from the biochemical analysis although this was not as clearly visualized in the histological preparations The rise in plasma TG suggests an increased output of esterified fatty acids from the liver as stated before the relation between plasma TG levels and liver TG content also highly suggests that the rate of this output was determined by the size of the liver TG pool In myocardium and skeletal muscle oxidation would be

the principal depletion mechanism it is noteworthy that the amount of stainable fat in muscle fibers at the end of these experiments appeared to be even smaller than at the beginning. The close topographical relation between mitochondria and lipid bodies in myocardium and skeletal muscle as revealed by electron microscopy may visualize the process of lipolysis and oxidation The same picture has been obtained by other authors in organs known to depend on oxidation of fat under the experimental conditions

The SDH reaction was markedly coarser in NA-infused animals Nitro-BT has been stated to be insoluble in lipid but the formazan may be adsorbed in excessive amounts on the surface of nonpolar lipid thus giving an artifactual distribution of the stain presumed to demonstrate the position of mitochondria (HITZEMAN 1963) That this was not the cause of the altered reaction is suggested by the fact that coarser deposition of formazan occurred in all cells regardless of lipid deposition FERRANS *et al* (1964) in a study of isoproterenol-induced cardiac necrosis in rats demonstrated that the same kind of reaction change was connected with mitochondrial damage However in the absence of any conspicuous and generalized ultrastructural changes in the mitochondria during excessive FFA mobilization, the coarser deposition of formazan in the SDH reaction might be tentatively interpreted as indicating enhanced activity possibly as an adaptation to increased demands for oxidation of fatty acids At any rate the failure of establishing any ultrastructural correlation to the altered size and distribution of formazan deposits is noteworthy and implies great caution when interpreting changes in the SDH reaction as due to gross cellular damage

The observations that the clinical symptoms—fever tachypnea and tachycardia, developing during the infusion of NA—persisted even after the infusion had been withdrawn, aroused the suspicion that depletion of rapidly increased lipid stores in cells might present a serious problem to the organism Oxidation of large amounts of fatty acids may have accounted for the increased temperature, in most animals the liver temperature was markedly increased Many myocardial and skeletal muscle fibers were edematous suggesting that the depletion process was not without consequences for normal structure Electron microscopy was however not made on this part of the material

The overload caused by the increased FFA flux may also have disturbed normal elimination processes e g in the lungs The

alveolar wall cells are now for many reasons held responsible for the elaboration of the lung surfactant They are esterase positive, the appearance of a positive esterase reaction in alveolar wall cells of fetal mouse lungs on the 17th to 18th day of gestation (WIRSÉN unpublished) coincides with that of a surface active substance (BUCKINGHAM and AVERY 1962) and also of the inclusion bodies characteristic of these cells in electron micrographs (WOODSIDE and DALTON 1958) It is probable that a major part of the fat laden cells described in Paper I were alveolar wall cells overloaded with fat This overload is apparently deleterious for their normal function and may be the underlying cause for the widespread atelectases after prolonged NA infusion These would then occur as a result of increased surface tension *(Plate 2)*

Conclusions

Expansion of the intracellular fat pools is proposed to be of major importance for regulating the rate of depletion of lipid stores in the cells by secretion or oxidation

The depletion process may cause disturbances of normal function and present serious problems for an organism subjected to excessive FFA mobilization

The increased size and altered distribution of formazan deposits in the reaction for succinic dehydrogenase may in the absence of any obvious ultrastructural change in the mitochondria be interpreted as due to increased oxidative activity The present findings emphasize that the staining reaction may not give an adequate picture of the state—or even distribution—of the mitochondria

CHAPTER IX

Fatty Acid Uptake and Storage in a Metabolically Heterogeneous Tissue (Skeletal Muscle)

Three types of muscle fiber are found in higher vertebrates red intermediate and white (for references see Papers IV and VI) The red fibers depend mainly on fat and the white mainly on carbohydrates for their energy expenditure, as the name implies intermediate fibers appear to depend on both Stainings for lipids and glycogen lend support to the concept that each type stores its appropriate fuel, as there are often fat droplets in the sarcoplasm of red fibers and glycogen in that of white The fact that skeletal muscle is one of the greatest, if not the greatest consumers of fatty acids in the body together with its being composed of three metabolically different cell types in the same extracellular space, made this tissue highly suitable for elucidating the problem whether or not there is a selective uptake of FFA by different cells

The development of metabolic heterogeneity during fetal life (Paper VI)

This paper presents the first report on the histochemical differentiation of skeletal muscle fibers in the trunk musculature of fetal and newborn mice

By phosphorylase staining it was shown that the fibers presumably corresponding to adult white fibers (high phosphorylase activity) can be clearly seen to develop and grow on the 16th gestation day Intermediate fibers developed as a new distinct population during the following days but it was not until the 19th day that future red fibers (very low phosphorylase activity) were seen in all stages of development At birth on the 20th to 21st gestation

day the musculature had assumed an adult appearance with respect to fiber form and phosphorylase staining However other histochemical parameters such as SDH content were not as easily distinguishable It was noted that the muscle spindles developed following essentially the same pattern as the fibers in the adjacent musculature

The results of this study appeared to provide a secure basis for interpreting the histochemical changes in skeletal muscle after excessive FFA mobilization as reflecting the reactions of three separate cell populations with a common blood supply

The relation of intracellular lipid deposition to histochemical parameters (Papers IV and V)

There were usually three degrees in the extent of lipid deposition in skeletal muscle fibers after enhanced FFA mobilization as seen in the preparations made for general survey In the series fixed with formol sucrose there was a good correlation between the amount of fat droplets and that of myoglobin in each of the fibers it may thus be concluded that significantly more lipid is deposited within red fibers Moreover the generally reciprocal relationship between fat and glycogen content seen in the cryostat series was also prominent in electron microscopic preparations Nonspecific esterase activity in fresh frozen specimens was present in fibers with high content of fat droplets as the same correlation prevails also in other tissues this enzyme reaction may reflect the presence of cellular mechanisms associated with the esterification of fatty acids *(Plate 3)*

The contention that lipid deposition may be related to inherent metabolic qualities had thus been corroborated However several fibers did not fit into the general pattern with respect to every staining reaction this has also been the impression of other authors (STEIN and PADYKULA 1962) Therefore a definite conclusion must await further study Moreover while the histochemical and electron microscopical findings highly suggest the possibility of a selective uptake of FFA in different cells what they actually demonstrate is that some cells store more lipid as droplets than others

To test the hypothesis that the deposition pattern in muscle and possibly in other tissues reflects a selective uptake of FFA one must follow the fatty acids themselves from plasma to cytoplasm This was done in the last paper of this investigation

Autoradiography of intravenously injected albumin bound 1 C^{14} palmitate in skeletal muscle (Paper VII)

This paper presents the first report on the autoradiographic distri-bution of labeled palmitic acid taken up from plasma

The study was made on pigeon pectoralis muscle for two mai reasons First, its histochemistry and biochemistry with special re ference to fat metabolism has been extensively studied (for refer ences, see Paper VII) Secondly the three types—only the red an the white being present in pectoralis major—constitute also thre distinct caliber classes, the white fibers being the largest Thus, wit the resolution power of apposition autoradiography—about 20 μ— identification of each fiber type would be feasible even withou supplementary histochemical stainings

Most of the radioactivity was found in red fibers and least in white Now, the labeled fatty acid may be present in four main fractions in the specimen namely, in plasma FFA, and intracellular diglycerides, TG or phospholipids (GORANSSON and OLIVECRONA 1964) Owing to the probably very short biological half life of plasma FFA in birds this fraction will be rapidly decreasing So will also the diglyceride fraction, as the diglycerides are converted to phospholipids or TG Thus, after the first few minutes label will be present mainly in bound or nonpolar lipids—mostly TG—in the sarcoplasm (GORANSSON and OLIVECRONA 1964) In spite of the red fibers having the greatest capacity for oxidation of fatty acids they displayed the highest label content this suggests that uptake of FFA was the main determining factor in the pattern obtained A selective uptake if not possibly a selective extraction of plasma FFA by metabolically different fibers in skeletal muscle now ap-peared to be established *(Plate 4)*

Conclusions

The three fiber types red intermediate and white in the skeletal muscles of higher vertebrates appear to develop as three distinct populations

After enhancement of FFA mobilization, the extent of lipid depo-sition appears to be related to certain histochemical parameters and

may be assumed to be generally characteristic of each fiber typ
reflecting its dependence on fat as source of energy

The different amounts of fat droplets appearing in cells aft
enhanced mobilization of FFA in all probability reflect a selectiv
uptake of FFA from plasma

Concluding Remarks

Lipid mobilization is an essential process in all organisms storing their energy in the carbon chains of fatty acids The aim of the present investigation has been to study some principal problems in this process by a morphological and histochemical approach in order to provide a structural basis for the interpretation of biochemical and physiological findings

The following contentions have emerged from these studies

1 Adrenergic nerve terminals in white adipose tissue are confined to the blood vessels mainly arteries and arterioles On the basis of this finding earlier concepts of a direct adrenergic innervation of white adipose tissue cells are rejected It is proposed that the 'tonic influence of the sympathetic nervous system on the mobilization of free fatty acids in noradrenaline responsive species may be exerted via noradrenaline circulating in plasma

Brown adipose tissue cells appear to be innervated by delicate terminals However, this tissue does not contribute appreciably to the plasma levels of free fatty acids but is an important site of heat production, thus, conclusions arrived at by experiments with brown adipose tissue (denervation etc) should not be indiscriminately applied in the discussion of lipid storage and mobilization in the white The fundamental difference between the two should be properly recognized

2 The different responsiveness to noradrenaline as a fat mobilizing agent in various species appears not to be reflected in the innervation pattern in white adipose tissue The local terminals around blood vessels may be regarded as mainly vasoregulatory

3 During excessive free fatty acid mobilization the intracellular deposition of lipid mainly in the form of triglyceride droplets is correlated to the increased levels of free fatty acids in plasma Changes in the other plasma lipid fractions notably the triglycerides are *not* observed to contribute significantly

4 Ultrastructurally, lipid is deposited in the free cytoplasm without conspicuous relation to cytoplasmic organelles or membrane systems The appearance of lipid as single large droplets suggests that enzymes involved in the esterification are located around the droplet

surface The lipid droplets are often seen in close proximity to mitochondria especially in myocardium and skeletal muscle, lipolysis may proceed at the interface to provide free fatty acids for subsequent oxidation by intramitochondrial enzymes

No information about the transport of fatty acids from plasma to the cells was obtained Enhanced free fatty acid mobilization appeared not to have caused any conspicuous damage to cell structures

5 Depletion of the newly formed lipid stores is observed on withdrawal of noradrenaline infusion in dogs as the plasma levels of free fatty acids rapidly return to baseline values Conceivable mechanisms would be oxidation of the fatty acids or—in liver and lung—secretion of lipoprotein In the lung however the normal elaboration of the lipoprotein rich surfactant may have been disturbed by the excessive FFA mobilization resulting in an overload of alveolar wall cells atelectases were frequent after prolonged infusion Forced depletion of rapidly increased lipid stores in the cells may present a serious problem also in other tissues and thus for the whole organism

It is suggested that increased mobilization of free fatty acids may influence the rate of depletion by adding to the size of the intracellular fat pools In view of the fact that only part of the mobilized fatty acids are immediately oxidized this may be the actual physiologic purpose

6 In skeletal muscle which is composed of three fiber types apparently developing as separate populations during fetal life lipid deposition is correlated i a to the myoglobin content red fibers store significantly more lipid than do intermediate and white Autoradiography of injected labeled palmitate supports the concept that the distribution of stainable lipid reflects a selective uptake of fatty acids from plasma according to the extent to which different cells use fat as energy source

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Computer simulation of ferrokinetic models

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JEAN CLAUDE VUILLE

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I Investigations on the compatibility of different models with experimental data

II Significance of diurnal and day to day variations of plasma iron concentration with respect to ferrokinetics

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CHAPTER I

Introduction

In vivo studies on iron kinetics are generally based on observations of the behaviour of intravenously injected radioiron in plasma Semi logarithmic plot of serial radioactivity measurements during a period of 1 or 2 weeks reveals several more or less distinct components in the disappearance pattern of the tracer which can be approximated by the sum of a few exponentials (cf Pollycove and Mortimer 1961) Sharney Wasserman Schwartz and Tendler (1963) Theoretically a multiexponential pattern may be explained either by the assumption of non homogeneity of the plasma iron compartment caused by the existence of several iron protein complexes with different binding characteristics or by the assumption of a feedback to the plasma from one or more pools of exchangeable iron Dern Monto and Glynn (1963) using a double label technique have given some evidence in favour of the former hypothesis whereas Hosain and Finch (1964) were unable to confirm these findings even the results of Dern et al would not be sufficient to explain quantitatively the observed behaviour of radioiron in plasma One is thus left with the other theoretical possibility the existence of feedback mechanisms In this case different models may be conceived consisting of different iron pools and flows connected to the plasma compartment in a specific manner The basic structure of such a model cannot be deduced from plasma radioiron data alone as several different arrangements of the various compartments would be able to explain these experimental data this general

statement is valid for all tracer experiments (cf Bergner 1962) Pollycove and Mortimer (1961) proposed a detailed model the basic feature of which is a labile iron pool interposed between the plasma compartment and that iron which is irreversibly fixed in haemoglobin In the argumentation in favour of this model qualitative aspects of surface measurements over the sacral bone marrow liver and spleen were taken into account but for the calculation of the actual size of the pools and flows only the plasma data could be used in some pathological cases complemented by a figure derived from the maximum utilization of the radioiron for haemoglobin synthesis In normal humans the values for daily haemoglobin synthesis computed on the basis of this model corresponded quite exactly to the figures predicted by the assumption of an erythrocyte life span of 120 days However this procedure has two drawbacks In the first place the general structure is conceived from quantitative measurements in one compartment only surface measurements are probably of very little value in this connection Secondly determination of the final slope in the plasma disappearance curve requires the injection of relatively high doses of radioactive iron and great care in handling the blood specimens as by the end of the second week nearly 100 % of the injected amount is present in circulating red cells and only about 0 1 % is left in plasma even slight haemolysis would thus lead to erroneously high activity in the plasma samples

In view of these drawbacks Garby Schneider Sundquist and Vuille (1963) suggested an approach which allowed a quantitative interpretation of the rate of radioiron incorporation into newly formed red cells In two artificial cases constructed from a normal material it could be shown among other things that this approach generates reasonable values for the most important parameter of ferrokinetics of the normal man namely the rate of haemoglobin synthesis

The present study was undertaken in order to test the validity of the model of Garby et al in individual cases where the plasma iron turnover (first slope in the plasma disappearance curve multiplied by the plasma iron content) and the appearance of the radioiron in circulating red cells were determined simultaneously During the course of this analysis however some inconsistencies in the original model became evident and it proved therefore necessary to introduce several modifications in order to arrive at a solution which *should fullfill the* following requirements

1 The model has to be compatible with the experimental results *obtained from both those compartments that offer the possibility*

for quantitative radioactivity assay (plasma and circulating red cells)

2 The basic structure of the model has to be in accordance with available experimental results other than those mentioned above and hypothetical features should be accessible to direct experimental investigation

3 The experimental data obtained from individual cases should determine the numerical values of the different parameters of the model in a unique manner and these values (sizes of pools and flows) should be in accordance with those obtained by independent methods If available experimental data do not suffice to fullfill this requirement of uniqueness the model should be able to predict which experimental measurements have to be performed in order to improve the uniqueness of the solutions

At present the following experimental data relating to ferrokinetics are available

1 Quantitative tracer data in plasma and red cells after an intravenous injection of radioiron (Pollycove and Mortimer 1961 Sharney et al 1963 Garby et al 1963 Hosain and Finch 1964)

2 Semiquantitative tracer data from surface measurements over liver spleen and bone marrow after an intravenous injection of radioiron (Pollycove and Mortimer 1961 Sharney et al 1963)

3 Data concerning the kinetics of erythroblast maturation in the bone marrow (summarised by Lajtha and Oliver 1960)

4 Measurements of the rate of radioiron incorporation into the haem of red cell precursors (Pollycove and Maqsood 1962 Najean Ardaillou and Mulmann 1964 Myhre 1964 Noyes Hosain and Finch 1964)

5 Measurements of non haem iron content in red cells (cf Faber and Falbe Hansen 1959)

6 Measurements of the erythrocyte life span yielding indirect estimates of haemoglobin production in steady state (cf Berlin 1964)

7 Results of studies on the mechanisms of haem breakdown (London West Shemin and Rittenberg 1950 Ostrow Jandl and Schmid 1962 Israels Skandenberg Guyda Zingg and Zipursky 1963 Nakajima Takemura Nakajima and Yamaoka 1963 Garby and Noyes 1959)

In the following analysis the compatibility of the different ferrokinetic models with the data 1 and 6 mentioned above will be considered in the first place as these data are at the same time the most important and the best established ones

The approach in this study is rather unconventional and largely empirical in many details. In contrast to the usual analytical approach ("multicompartment analysis"), this method has the advantage of higher flexibility which allows the investigation of quite complex models with rather simple mathematical tools. Concerning the models studied in the present work, no straightforward proof for compatibility (or incompatibility) with experimental data can be given and therefore all models that have been analysed will be presented in detail.

According to Pollycove and Mortimer (1961) plasma radioiron data in the normal man can be approximated fairly well by the sum of two exponentials only. This puts some restrictions on the general three compartment model of Garby et al. (1963) in the sense that feedback to plasma has to be assumed to occur mainly from one of the two labile pools, either from the lateral "storage" pool or from the pool interposed between plasma and the haem iron of the red cell precursors. The decision in favour of one of these two alternative concepts cannot be made a priori. Therefore both these special cases of the model of Garby et al. will be analysed under the headings of *model I* and *model II* respectively.

CHAPTER II

Material and Methods

1 *Experimental*

Radioiron experiments were made in eight clinically healthy adult volunteers. One of them (case 8) had donated 400 ml blood 2 weeks before the beginning of the study and during the first two weeks of the experiment her haemoglobin concentration rose from 11.9 to 12.6 g %. Throughout the experimental period her reticulocyte count was slightly above normal (mean 1.8 %), thus there was some suspicion of slightly increased erythropoiesis during the experimental period. The other seven cases were considered to be haematologically normal as judged by haemoglobin concentration, haematocrit and serum iron concentration. The pertinent data are included in table I.

All experiments started in the morning between 8 and 10 a.m. 16 ml of blood were withdrawn from a vein and transferred to a sterile flask containing 4 ml ACD solution as an anticoagulant and 5 to 8 µc Fe^{59} citrate of high specific activity (more than 3 µc/µg). The samples were centrifuged at 1,500 g for 10 minutes and stored at 3 °C for 20 to 30 minutes in order to ensure a firm binding of the iron to the plasma transferrin prior to injection. After the incubation 5 to 10 ml of the plasma were reinjected and 4 blood specimens were withdrawn from another vein during the following 3 to 5 hours. The blood was collected in heparinized tubes and centrifuged at 1,500 g for 10 minutes. Thereafter 2 to 4 ml plasma samples were counted for radioactivity in a well type scintillation counter. The counting time was adjusted so as to reduce the random counting error to 1.5 % or less. In cases 1—6 and case 8 the activity in plasma 1 day after injection was measured as well, but for these samples the random error was much higher because of very low activity. From the first 3 or 4 measurements (samples obtained more than 4 hours after injection were not included) the initial slope of the plasma disappearance curve was calculated by the method of least squares. This estimate together with its standard error is shown in table I for all cases. The 24 hours estimates are included as well in table I.

Serum iron determinations were performed in duplicate by the method of Heilmeyer and Plotner (193) as modified by Agner (194) at a time when approximately 50 % (?8 to 65 %) of the injected radioiron had disappeared from the circulation.

For the determination of the amount of radioiron incorporated in circulating red cells 5 blood samples were withdrawn during the first week after injection, 3 samples during the second week and 2 samples during the third week. Except for the first and second day samples whole blood was used for radioactivity measurements, as from the third day on the contribution of plasma radioiron to

case nr	age (years)	sex	weight (kg)	Hb conc (g/100 ml)	haematocrit	serum iron conc (μg/100 ml)	Fe^{59} injected (μc)
1	21	m	72	16 1	0 49	68	4 0
2	29	f	60	12 6	0 41	85	3 0
3	24	f	59	13 5	0 41	77	3 0
4	30	m	60	15 6	0 46	147	5 0
5	20	f	62	12 9	0 38	152	3 0
6	26	m	71	13 5	0 40	120	5 0
7	30	m	71	15 3	0 43	137	3 5
8*	26	f	65	12 4	0 39	120	1 0

case nr	calculated blood volume (ml)**			total Hb mass (g)	plasma iron content (mg)	initial slope of Fe^{59} disappearance ± s e (d^{-1})	radioactivity in plasma 1 day after inj ± s d (%)
	total	plasma	red cell				
1	5400	2960	2440	799	2 01	14 2±0 35	0 90±0 71
2	4900	2690	1580	487	2 23	10 0±0 20	0 87±0 36
3	3640	2270	1370	452	1 75	9 1±0 05	0 93±0 38
4	4500	2600	1900	648	3 82	7 6±0 15	0 54±0 07
5	4840	2890	1590	516	4 29	6 6±0 44	1 36±0 0[illegible]
6	5330	3370	1960	662	4 04	6 3±0 19	0 6[illegible]
7	5330	3220	2110	732	4 41	5 6±0 08	
8*	4550	2920	1630	519	3 50	16 5±0 17	

Table I

Some clinical data and experimental results of the e[illegible] tals

* Case 8 had donated blood (400 ml) two weeks [illegible] g[illegible] study

** Calculated on the basis of 75 ml/kg for men a[illegible] plasma and red cell volume from total bloc[illegible] was multiplied by the factor 0 92 (cf Dono[illegible]

the total activity in whole blood is negligib[illegible] precision is increased by reducing the num[illegible] collected in heparinized glass tubes and the[illegible] in an International Microcapillary Centrifuge made according to Garby and Vuille (1961) by adding a small amount of saponin pow[illegible] tubes weighed again and counted for radi[illegible] rections were made for geometry using a [illegible] Fe^{59} standard solutions The total volume of calculated using the formula

$$\frac{u\ Hct}{[illegible]\ 06}$$

u = weight of ha[illegible]

Hct = haematocrit [illegible]

The factor 1.06 for the specific weight of whole blood was taken from Van Slyke et al (1950) The difference in haemoglobin concentration in the various samples was so slight that it would not have been able to change the specific weight of the whole blood significantly and therefore the same factor was used for all samples

The fraction of the injected radioiron present in red cells was calculated as follows The activity in plasma (*cpm/ml*) as a function of time after injection was plotted on semilogarithmic paper a straight line fitted to the single points and extrapolated back to time $t=0$ The intercept at $t=0$ was taken as 100%. From this figure the percent of radioiron present in circulating red cells was calculated by the formula

% radioiron present in circulating red cells = C

$$C = \frac{(\text{cpm/ml RC})\ (\text{total red cell volume})\ 100}{(\text{cpm/ml plasma at } t=0)\ (\text{total plasma volume})}$$

$$C = \frac{(\text{cpm/ml RC})\ \text{Hct}\ 0.92\ 100}{(\text{cpm/ml pl at } t=0)\ (1-\text{Hct}\ 0.92)}$$

The factor 0.92 was chosen in order to correct the venous haematocrit for the true body haematocrit (Donohue Motulsky Giblett Pirzio-Biroli Viranuvatti and Finch 1955) This factor may be false by some ± 5% and thus contributes a great deal to the overall experimental error This uncertainty could perhaps be reduced somewhat by simultaneous determination of both plasma volume and red cell mass by independent methods but even with very sophisticated techniques an experimental error of about ± 3% would be unavoidable and this improvement does not motivate the extra load with radioactive isotopes which such measurements would require

About half of the determinations were performed in duplicate From 45 duplicate determinations a standard error of the estimate of 2.33% was calculated for the case of a single determination For duplicate determinations which were performed mainly in the samples taken 8 or more days after injection the standard error was 1.65% The appearance curves of the eight cases are shown in fig 1

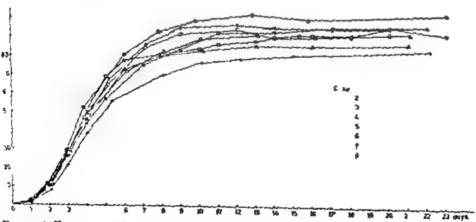

Figure 1 Experimental appearance curves of eight healthy adults

case nr	age (years)	sex	weight (kg)	Hb conc (g/100 ml)	haematocrit	serum iron conc (µg/100 ml)	Fe^{59} injected (µc)
1	24	m	72	16.1	0.49	68	4.0
2	22	f	60	12.6	0.41	85	3.0
[illegible]	24	f	52	13.5	0.41	77	3.0
4	30	m	60	15.6	0.46	147	5.0
5	26	f	62	12.9	0.38	152	3.0
6	25	m	71	13.5	0.40	190	5.0
7	30	m	71	15.3	0.43	137	3.5
8*	26	f	65	12.4	0.39	120	1.0

case nr	calculated blood volume (ml)**			total Hb mass (g)	plasma iron content (mg)	initial slope of Fe^{59} disappearance ± s.e. (d^{-1})	radioactivity in plasma 1 day after inj ± s.d. (%)
	total	plasma	red cell				
1	5400	2960	2440	799	2.01	14.2±0.35	0.96±0.41
2	4200	2620	1580	487	2.23	10.0±0.20	[illegible].87±0.36
3	3640	2250	1350	452	1.75	9.1±0.05	[illegible].93±0.38
4	4500	2600	1900	648	3.82	7.6±0.15	0.53±0.07
5	4340	2870	1520	516	4.29	6.6±0.44	1.36±0.05
6	5330	3350	1960	662	4.04	8.3±0.19	[illegible].66±0.05
7	5330	3220	2110	752	4.41	5.6±0.09	—
8*	4550	2920	1630	519	3.50	16.5±0.17	1.99±0.90

Table I

Some clinical data and experimental results of the eight individuals studied

* Case 8 had donated blood (400 ml) two weeks before the beginning of the Fe^{59} study

** Calculated on the basis of 75 ml/kg for men and 70 ml/kg for women. In calculating plasma and red cell volume from total blood volume the venous haematocrit was multiplied by the factor 0.92 (cf. Donohue et al. 1955)

the total activity in whole blood is negligible and obviously the experimental precision is increased by reducing the number of manipulations. The blood was collected in heparinized glass tubes and the haematocrit determined in duplicate in an International Microcapillary Centrifuge. Correction for trapped plasma was made according to Garby and Vuille (1961). The blood was then haemolysed by adding a small amount of saponin powder, transferred to preweighed plastic tubes, weighed again and counted for radioactivity as the plasma samples. Corrections were made for geometry using a standard curve obtained by means of Fe^{59} standard solutions. The total volume of the red cells in the sample was calculated using the formula

$$V^{RC} = \frac{w \cdot Hct}{1.06} \text{ ml}$$

w = weight of haemolysed whole blood (gm)
Hct = haematocrit corrected for trapped plasma

necessarily be equal to chemically defined compartments of the same size. But even purely "mathematical" pool sizes without direct physiological implication may have some significance when one is to compare the figures obtained in different individuals or in normal and pathological states.

3. The whole system is assumed to be in a steady state, i.e. sizes of pools and flows of non-labelled iron ("mother substance") do not change during the time of the experiment. The validity of this assumption, especially concerning the well known diurnal variation of plasma iron concentration, will be discussed in a forthcoming publication (Vuille 1965). Suffice it to say that periodical deviations from a perfect steady state will not materially influence the conclusions drawn in this study.

In a system where assumptions 2 and 3 are valid, the transfer rate of the tracer from one compartment to the other at any time t is directly proportional to the amount of tracer present at that time in the "starting" compartment:

$$J^*_{ij}(t) = X^*_i(t) \cdot k_{ji}$$

where $J^*_{ij}(t)$ is the flow of tracer from compartment i to compartment j at time t and $X^*_i(t)$ is the amount of tracer present in compartment i at time t. For the mother substance then the general equation

$$J_{ij} = X_i \cdot k_{ji}$$

is valid, i.e. from determination of the flow constants k_{ji} by tracer experiments the actual flows of mother substance as a function of compartment size can be calculated. In a multicompartment system a set of simultaneous differential equations of the form

$$\frac{dX^*_i}{dt} = -X^*_i(t) \cdot k_{1i} - \dots - X^*_i(t) \cdot k_{iT}$$

describing the changes of tracer amount in any one compartment at any time t can be set up, and these simultaneous equations may be integrated yielding analytical expressions for the amount of tracer as a function of time in each compartment. In the work of Garby et al. (1963) such analytical expressions were presented describing the radioiron disappearance curve in plasma and the radioiron appearance curve in circulating red cells. These expressions are of course only valid for models of the same basic structure as that of Garby et al. and any alteration of this configuration would change the set of differential equations that have to be integrated. When more complicated models have to be analysed the mathematical difficulties rise rapidly

and for the present work it was therefore decided to chose the more flexible method of direct numerical integration. In the analysis of the disappearance curve the use of analytical expressions has the great advantage that the numerical values of the parameters in these expressions can be deduced directly from the experimental data by the simple procedure of curve "peeling", especially if the different components are sufficiently separated from each other. Such a procedure is impossible in the case of the appearance curve (cf. Garby et al. 1963). In order to determine the numerical values of the parameters from these experimental data a fitting procedure using a digital or an analogue computer is necessary. If the restrictions which can be made *a priori* upon the model are rather weak, it would be desirable to be able to vary the rate constants of the model directly in such a fitting procedure. Otherwise it is very difficult to prove the uniqueness of the obtained solutions. If several solutions are possible the final solution obtained by an automatic fitting procedure may be unduly influenced by the fitting method which is chosen. Unfortunately, the parameters in analytical expressions describing the behaviour of the tracer in complicated systems (three compartments or more) cannot be computed from rate constants given *a priori*: calculations can only be done in the opposite direction, from analytical parameters to rate constants. In a numerical integration procedure, however, the rate constants appear as such in the necessary equations. This is a great advantage when the question of possible non-uniqueness of the obtained solutions arises. Moreover, if one wishes to analyse several models of different basic structure, the method of direct simulation by numerical integration is much more flexible than the analytical method which requires a complete reformulation each time a detail of the model is changed.

In the numerical integration procedure chosen for the present work (first order), the basic relation

$$\frac{dX^*_i}{dt} = -X^*_i(t) \cdot k_{ij} - \ldots + X^*_j(t) \cdot k_{ji} + \ldots$$

is the only necessary mathematical expression. However, the differential quotient $\frac{dX^*}{dt}$ has to be replaced by the quotient of the differences $\frac{\Delta X^*_i}{\Delta t}$.

The length of the time interval Δt may be chosen arbitrarily, but the approximation to the true differential quotient becomes more and more accurate with smaller and smaller Δt.

In digital computer "experiments" using this technique the events in the hypothetical system are simulated step by step symbolising the total amount of tracer injected into the plasma compartment at time $t=0$ by the value 1.0. Subsequently this amount is distributed step by step between the different compartments according to rate constants which are introduced as input data by the operator. As a result the fraction of the total injected tracer amount present in any compartment at any time t is obtained.

As mentioned above, the length of the step Δt determines the goodness of the approximation to the true differential quotient and therefore a very small step should be chosen for high accuracy. On the other hand, reduction of Δt increases the computer time which is required to simulate an experiment of given duration, and therefore a compromise has to be made between the desired accuracy on one hand and the available computer time on the other hand.

Another important question is how to fit the theoretically produced curves for radioiron concentration in plasma and circulating red cells to the experimental curves. Usually the best fit is defined by statistical criteria (method of least squares). In complicated systems, however, one has generally to admit the existence of more than one possible solution and the statistically best solution may not be the best solution from the physiological point of view. The experimental error may be different in different parts of the experiment; this difficulty could be overcome to some extent by "weighting" the deviations in the different points. But still there remains the difficulty that variation of a physiologically quite unimportant parameter may give rise to a significant deviation in a region of the curve where the experimental precision is high, and in this case the influence of this unimportant parameter on the choice of the statistically best solution would be unduly high. It was therefore decided not to define the "compatibility" of a certain model with the experimental data in statistical terms but to leave this decision in every case to the visual evaluation of the operator. The question how well the various rate constants in a compatible model are defined by the experimental data and which experimental points are most important will be treated in a forthcoming paper (Groth, Sandevall, Schneider and Vuille 196?).

During the course of this work much could be learned about the influence of single parameters on the different parts of the curves and it would not have been possible to translate this knowledge into a machine program for the type of computer used in this study. Therefore not only the evaluation of compatibility (which of course can

be objectivised afterwards by calculating the mean deviation of the theoretical from the experimental values and comparing this deviation with the experimental error) but also the successive variation of the rate constants was done by the operator. By this procedure an acceptable fit could be obtained by the production of some ten curves only.

The computer experiments in this work were programmed in FORTRAN I and run on an IBM 1620 with a storage capacity of 20 000 positions. The program for the first model tested will be discussed in some detail in chapter III and for the following models only the altered parts of the program will be presented.

CHAPTER III

Model Ia

1 *Presentation of the model and the corresponding computer program*

Model Ia is depicted in figure 2. Its basic structure is similar to that of the model of Garby et al. (1963) with two labile compartments being in exchange with plasma iron. By definition, however, model Ia represents that special case of the general three compartment system in which exchange with compartment S is *a priori* considered to be small as compared to the exchange with compartment M. As will be shown later, a complete reduction to a two compartment system did not allow an acceptable fit of all experimental appearance curves and therefore the basic three compartment structure is retained. In the

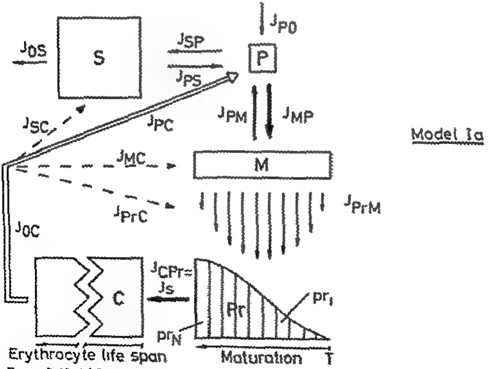

Figure 2 Model Ia

work of Garby et al. (1963) the rate of iron uptake by the red cell precursors was assumed to be uniform throughout maturation. This simplifying assumption is abandonned in the present model and a parabolic distribution of uptake rates is adopted as being more realistic (see p. 20). For most purposes of this study it is assumed that all iron return from senescent red cells occurs to the plasma compartment only, i.e. $J_{SC}=J_{MC}=J_{PrC}=0$ and $J_{PC}=J_{0C}$. This assumption has been discussed in detail by Garby et al. (1963). The following relations between pools, flows and rate constants are then valid.
P has to be determined by independent methods.

$J_{SP}=P\cdot k_{SP}$	$J_{MP}=P\cdot k_{MP}$
$S=J_{SP}/(k_{PS}+k_{0S})$	$M=J_{MP}/(k_{PM}+k_{PrM})$
$J_{PS}=S\cdot k_{PS}$	$J_{PM}=M\cdot k_{PM}$
$J_{0S}=J_{SP}-J_{PS}$	$J_{PrM}=J_{MP}-J_{PM}$
$J_{P0}=J_{0S}$	$J_{CPr}\equiv J_S=J_{PrM}$
	$J_{PC}=J_{0C}=J_S$

The program formulae for the amount of tracer in the different pools as a function of time *t* are then as follows (the superscript + which in chapter II was used to indicate that the formulae concern tracer are left aside in the following symbols). At the very beginning of the run *P* is set at 1.0 and $S=M=Pr=C=0$ and the time variable $t=0$.
t is now increased by Δt and the following calculations are performed.

$S_t=S_{t-\Delta t}+(P_{t-\Delta t}\cdot k_{SP}-S_{t-\Delta t}\cdot(k_{0S}+k_{PS}))\cdot\Delta t$
$P_t=P_{t-\Delta t}+(S_{t-\Delta t}\cdot k_{PS}+M_{t-\Delta t}\cdot k_{PM}-P_{t-\Delta t}\cdot(k_{SP}+k_{MP}))\cdot\Delta t$
$M_t=M_{t-\Delta t}+(P_{t-\Delta t}\cdot k_{MP}-M_{t-\Delta t}\cdot(k_{PM}+k_{PrM}))\cdot\Delta t$
$Pr_t=Pr_{t-\Delta t}+M\cdot k_{PrM}-pr_N$
$C_t=C_{t-\Delta t}+pr_N$

Pools *Pr* and *C* deserve some further comment. The pool *Pr* is divided into a number of small subcompartments pr_i. This number N is given by $N=T/\Delta t$, where *T* designes the whole period during which a given precursor is supposed to take up iron before leaving the bone marrow. The subcompartments *pr* symbolise the whole population of precursors in one and the same maturation stage. Besides taking up iron from the pool *M* at every time interval $t+\Delta t$ according to a parabolic distribution of uptake rates as a function of precursor age, these subcompartments *pr* have to be transferred from one location to the next at the end of each interval, thus simulating maturation. In the computer program this is effectuated by replacing pr_i by pr_{i+1}. At the end of maturation each subcompartment adds its accumulated radioiron to the pool *C*. pr_N symbolising the amount of radioiron present in the subcompart-

ment just prior to leaving the bone marrow. C accumulates all the radioiron it gets from Pr, as it is assumed that no red cells are destroyed within the time of the experiment (3 weeks). When all these calculations have been performed, t is increased again by Δt and calculations are performed in the same order as above. This procedure is repeated until t has reached a preset value of t_{max} or until calculations are interrupted by switch control.

2. *Some results of general importance*

In the following sections some results of general importance will be presented which were obtained mainly by analysing model Ia. Most of these results, however, are also valid — except minor modifications — for the subsequent models.

a) *The accuracy of the numerical integration method as a function of the length of the interval Δt*

As was pointed out in chapter II the approximation introduced by the substitution of the differential quotient dX_i/dt by the quotient of

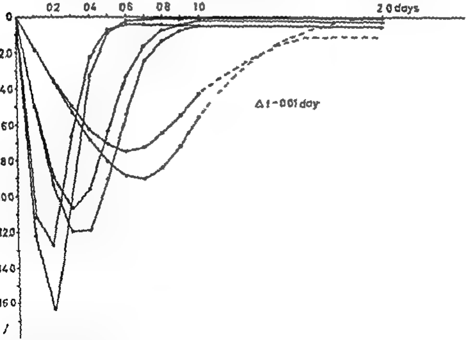

Figure 3. Percent error in the theoretical disappearance curves produced by the method of numerical integration of the first order as a function of time after injection. $\Delta t = 0.01$ day.

differences $\Delta Y_i/\Delta t$ is dependent upon the length assigned to the interval Δt. In order to test this dependency quantitatively six different combinations of parameters together with varying lengths of maturation time T were fed into the original program of Garby et al (1963) (analytically solved differential equations). The corresponding rate constants were calculated from these parameters and the radioiron curves for compartments P and C were generated. The same combinations of rate constants were then fed into the actual simulation program where of course for this special purpose the uptake rate in compartment Pr was constant throughout maturation together with different values of Δt (0.005, 0.01, 0.02, 0.05 and 0.1 day respectively). The disappearance and appearance curves generated by both methods for a given combination of rate constants and a given length of maturation time T were compared and the deviations at different points of the numerically integrated curve from the analytically integrated one expressed as percent error. In fig. 3 the error at different points of the disappearance curve is shown for $\Delta t = 0.01$ day. It can be seen that the deviation is always negative and greatest between $t = 0.2$ and $t = 0.7$ day, thereafter decreasing more or less rapidly. This decrease of the error is mainly due to the feedback from labile pools which

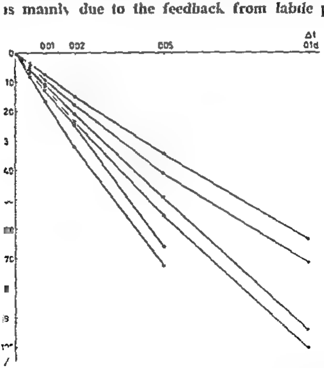

Figure 4 Maximum percent error in the theoretical disappearance curves produced by the method of numerical integration of the first order as a function of interval length Δt

leads to a successive correction of the initial error Fig 4 shows the maximum error in each case as a function of the interval length For $\Delta t=0.1$ day the maximum error was more than —100 % in some cases (actually negative values) but even in these cases did a rather rapid correction take place after $t=1$ or 2 days Fig 5 shows the error in different points of the appearance curves for $\Delta t=0.1$ day At $t=1$ day the error was between —6 and —13 % but decreased very rapidly to values of less than 1 % A simulation of a 3 weeks experiment with $\Delta t=0.1$ day required approximately half an hour on the IBM 1620 By decreasing Δt to 0.05 day the computer time rose significantly more than a factor 2 as not only the number of time intervals was doubled but also the number of subcompartments pr_i which had to be treated during each time interval In view of the fact that the value in the appearance curve at time $t=1$ day was mainly dependent on a single part of the precursor model (i e the distribution of uptake rates) and had practically no influence on the determination of the rate constants an error of about 10 % in this region was considered to be well

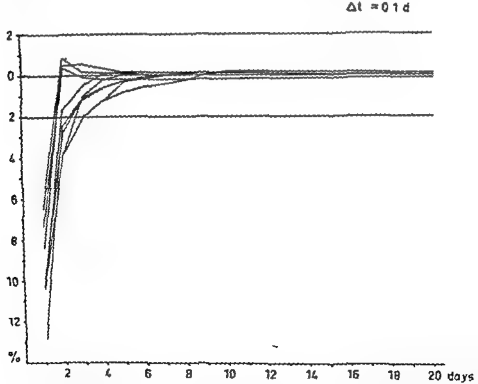

Figure 5 Percent error in the theoretical appearance curves produced by the method of numerical integration of the first order as a function of time after injection $\Delta t=0.1$ day The shaded area corresponds approximately to the experimental error

tolerable and therefore a Δt of 0.1 day was generally used in the fitting of the appearance curve. In cases where an accurate simulation of the first part of the disappearance curve was desired Δt of 0.002 or 0.001 day was used.

b) *The influence on the appearance curve of variations of the maturation time T and of the distribution of iron uptake rates during precursor maturation in the bone marrow*

The characteristic sigmoid shape of the appearance curve is the product of two main factors. The multiexponential type of the disappearance curve suggests the existence of a feedback of radioiron which had left the plasma compartment during the first hours, thus leading to some delay in the incorporation of the isotope into erythroblast haem. The other important factor is the time of maturation (T) of the erythroblasts within the bone marrow. This period T is defined in the model as the time during which an individual red cell precursor is supposed to take up iron. T thus includes all morphologically recognisable cells from the pronormoblasts to the bone marrow reticulocytes. In the earliest stages (pronormoblasts) the rate of uptake is probably quite low for the population as a whole, as there are relatively few cells in this stage of development. With proceding maturation and successive mitoses the uptake rate per maturation stage increases to a maximum and decreases thereafter again as no further mitoses occur and cell metabolism gradually slows down. This concept has been presented in more detail by Lajtha and Oliver (1960). The fact that circulating reticulocytes also take up some of the injected radioiron is neglected in this model as this is of no quantitative importance in normal cases. Mathematically, an initial increase up to a maximum followed by a decrease may as a first approximation be described by two straight lines, but in view of the fact that a continuous function is easier to handle, a parabolic course was adopted in the first part of the present work (fig. 2). The parameters a, b and c in the expression $y=a+bx+cx$ were determined as follows: a was generally set at zero, which means that iron uptake was assumed to be zero at the point of delivery to the circulation; b and c were determined by the length assigned to T ($y=0$ when $x=T$) and by the requirement of the integral $\int_0^T y\,dx$ being 1.0, as y describes the *relative* rates of iron uptake. For some special purposes a special computer

program was used where the relative uptake rates could be assigned arbitrarily to each precursor stage Fig 6 shows the change in the shape of the appearance curve which is brought about by assuming a parabolic type of uptake instead of a uniform one as in Garby et al (1963) In each subfigure a) and b) the rate constants k_{ji} are identical As can be seen (fig 6 b left curve) the distribution of the uptake rates during maturation has relatively little influence on the shape of the appearance curve in the case with a relatively high ratio M/P and a relatively short maturation time T the delay in the incorporation being caused mainly by dilution of the isotope within the pool M In the case of a small ratio M/P (fig 6 a) and or great T (fig 6 b right curve) however the distribution of the uptake rates in the marrow becomes more important In the extreme case with instantaneous labelling of the precursors the derivative of the appearance curve would exactly reflect the distribution of the uptake rates

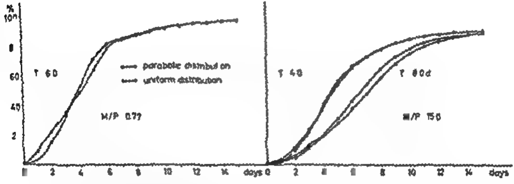

Figure 6 a *Figure 6 b*

Effect on the theoretical appearance curve of parabolic versus uniform distribution of uptake rates during precursor maturation

M/P	40		20		10		5		0 5	
T	Q1	Q2	Q1	Q2	Q1	Q2	Q1	Q2	Q1	Q2
3 0	97	87	1 08	74	1 35	.55	2.00	30	4 8	69
4 0	68	96	9	85	89	68	1 07	46	1 44	27
5 0	65	1 18	69	1 03	76	96	8[illegible]	81	1 02	66
6 0	63	1 31	65	1 22	70	1 10	79	99	90	.89
8 0	57	1 43	61	1.35	65	1 24	72	1 14	.80	1 07

Table II

Limitations in variability of the maturation time T by the single experimental criterion of maximum increment in the appearance curve being found consistently between days 3 and 4 (Q1 and Q2<1 0 see text)

If it is assumed that the uptake rates as a function of maturation stage are distributed symmetrically around the maximal value as represented by a parabolic curve some conclusions concerning the length of the total maturation time T may be drawn directly from the experimental data In all eight cases studied the maximum daily increment in the appearance curve was observed between days 3 och 4 This fact may be expressed in the following manner Define quotients $Q1 = (C_{-3})/(C_{3-4})$ and $Q2 = (C_{4-})/(C_{3-4})$ where C_{m-n} is the increment between days m and n Both $Q1$ and $Q2$ are less than unity if the maximum increment is $C_{3\ 4}$ Actually in the eight cases studied $Q1$ was 0 74—0 89 and $Q2$ was 0 68—0 89 5 different combinations of rate constants representing different magnitudes of delay within the labile pools together with T—values of 3 4 5 6 and 8 days respectively were fed into the computer program for models Ia and IIa (cf p 30) and the resulting appearance curves were analysed for the quotients $Q1$ and $Q2$ The results are presented in table II where the heavy line surrounds the area within which both $Q1$ och $Q2$ are less than 1 0 as required by the above mentioned experimental results Thus *for a given combination of rate constants especially a given ratio M/P* the range of possible T values is rather limited by this single criterion of $Q1$ and $Q2$ being less than unity

c) *The influence of variations of the different rate constants in model Ia on the characteristics of the appearance curve*

The most important characteristics of the normal appearance curve can be described by the three entities shown in fig 7 Slope 1 is the (maximum) increment between days 3 and 4 slope 2 the increment between days 10 and 15 divided by 5 With these two slopes and the percent incorporated radioiron on day 15 (C_{15}) the appearance curve is fairly well characterised

In order to study the influence of the different rate constants k_{ij} on each of these "reduced data a total of 640 different combinations of rate constants with systematic variation (all values of each rate constant against all values of all other rate constants) were analysed In order to reduce the number of curves that had to be produced some restrictions were made Thus the total plasma iron turnover was not varied (for all curves a value of [illegible] times plasma iron content per day was used) as this entity is determined by the first slope in the disappearance curve In other words the sum $k_{SP}-k_{UP}$ was always 10 0 and only the ratio k_{SP}/k_{UP} was varied Also the precursor part

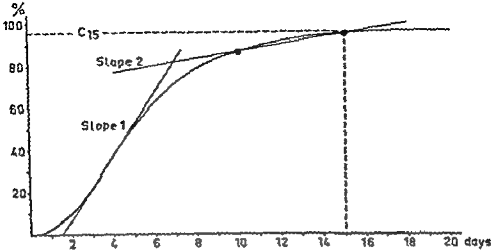

Figure 7 Reduced data drawn from a normal appearance curve

of the model was unchanged with a maturation time of 4 days and a parabolic distribution of uptake rates Furthermore J_{0S} was not taken into account In the ensuing fitting procedure in individual cases with other values of plasma iron turnover no exception in the general trend of the relations between rate constants and reduced data were encountered and concerning the maturation time T the general rules outlined in section 2 b) of this chapter (p 22) were taken into account The results of the systematic variation are shown in fig 8 Each subfigure represents only the general trend from a total of about 100 different regression lines In the two instances where no definite trend was observed (regression of slope 2 on λ_{PU} and on $\lambda_{UP}/(\lambda_{UP}+\lambda_{SP})$ respectively) all possibilities are depicted In all other cases no exception of the general trend was encountered

d) *Some observations concerning the uniqueness of the solutions obtained on the basis of model Ia and the present experimental data*

In the first fitting experiments only the first slope of the disappearance curve and the whole appearance curve were used Quite soon however it was recognised that a large number of different combinations of rate constants were able to fit these experimental data Especially the range for possible values of the flow J_S was extremely wide corresponding to a relative rate of haemoglobin synthesis of

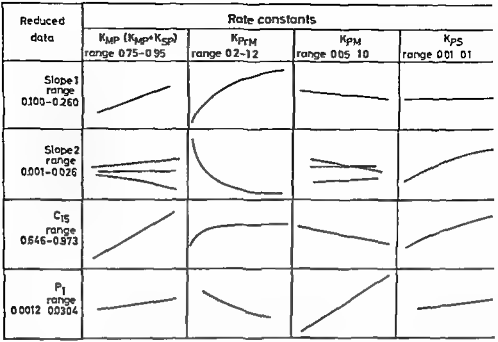

Figure 8 Influence of variations of the rate constants of model Ia on reduced dat (cf fig 7)

approximately 0 2 to 1 3 % of circulating haemoglobin mass per day The appearance curves corresponding to these possible solutions differ ed only slightly from each other so that a decision in favour of one or another of these solutions was not possible in view of the experimental error If the fitting procedure had been done automatically on the basis of the method of least squares one "best solution would probably have resulted and the high variability would have escaped attention When looking at the corresponding theoretical disappearance curves it was noticed that the "*final slope was almost identical for all these* possible solutions for one and the same individual but there was a marked difference in the situation of these slopes with respect to the y axis (see fig 9) When the radioactivity in plasma at time $t=1$ day (P_1) was taken in account however the range of possible solutions became very narrow as the y axis intercept of the second slope was now determined by the P_1 value The experiments in cases 1—4 had been performed before this important point had become clear and their P_1 values had been measured only approximately with a coeffi cient of variation of roughly 50 % Therefore it seemed worthwhile to look at the mathematical implications of the P_1 value in some more

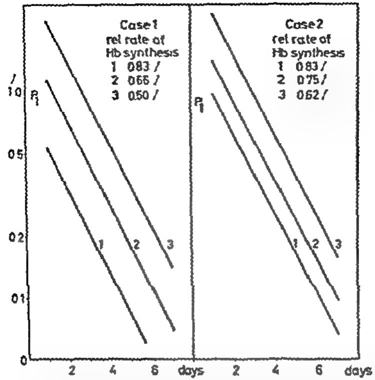

Figure 9 Theoretical disappearance curves after $t=1$ day produced on the basis of model Ia and with rate constants that yielded a good fit of the appearance curves of cases 1 and 2 Note the parallelity of the respective slopes Ordinate Percent of injected dose in plasma

detail According to Nosslin (1962) and to Bergner (1964) the turnover of an open system as a whole — irrespective of its structure — is determined by the reciproque of the integral under the tracer curve of that compartment into which the tracer is introduced In the case of the present model the turnover of the system as a whole is represented by $J_S + J_{0S}$ i e mainly J_S as J_{0S} is negligible when compared to J_S For the following consideration we will chose the simple two compartment model $\rightarrow P \rightleftarrows M \rightarrow Pr \rightarrow$ the corresponding disappearance curve of which can be described by the formula

$$y = H_1\, e^{-g_1 t} + H\, e^{-g t} \quad \text{where } H = 1 - H_1 \text{ and the integral}$$

$$\int_{t=0}^{t=\infty} y\, dt = \frac{H}{g_1} + \frac{1-H_1}{g}$$

(see fig 10)

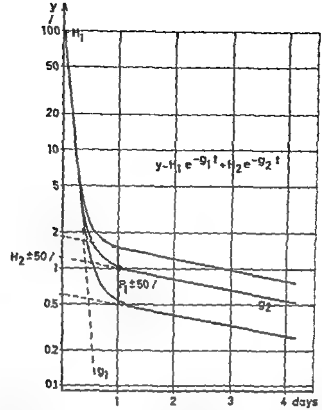

Figure 10 Importance of the P_1 value (radioactivity in plasma 1 day after injection) for the determination of the total turnover of a 2 compartment system (see text)

The integral is completely determined if the three independent variables g_1 g_2 and either H_1 or H_2 are known g_1 is determined by the initial slope and has a relatively small experimental error H_2 is in this system practically determined by P_1 and g_2 (see fig 10) P_1 is measured directly Concerning the variable g_2 the parallelity of the final slopes in fig 9 indicates that direct measurement of this variable in plasma may be replaced by fitting the parameters of a given model to the appearance curve and it seems that the error of estimating g_2 in this way is quite small The present method of "manual" adjustment of the parameters during the fitting procedure does not allow to draw quantitative conclusions about this point In any case the major contribution to the overall error in estimating the total turnover of the system is due to the error of the estimate of P_1 From fig 10 it is evident that the error of H_2 is proportional to that of P_1 The error of the estimate of the integral $\int_0^\infty y\,dt$ however will be less than the

error of the estimate of H In the present system the two parts of the sum on the right hand of the above equation are of the same order of magnitude and overestimating H would increase only the second part of the sum whereas the first part is unchanged or slightly decreased ($H_1 = 1 - H$) This finding indicates that even an approximate measurement of P_1 might allow a relatively high precision in the determination of the total turnover of the system Taking as an example the normal value for the parameters g_1 g and P_1 from Pollycove and Mortimer (1961) and assuming an experimental error of the P_1 estimate of $\pm 50\,\%$ the resulting error in estimating the total turnover would be $-16\,\%$ and $+23\,\%$ respectively

3 *Results of the fitting procedure on the basis of model Ia*

As a first step in the fitting procedure the first slope of the disappearance curve was fitted by adjusting the sum $k_{SP}+k_{MP}$ as the other rate constants have practically no influence on this slope During the subsequent fitting of the remaining experimental data this sum was not changed A more or less arbitrary combination of k_{ij} was now fed into the computer and the resulting appearance curve and P_1 value compared with the corresponding experimental data With the help of fig 8 one or more rate constants and/or T were varied in order to obtain a better fitting The procedure was repeated several times until the theoretical curve followed the experimental one within a range corresponding approximately to the experimental error All the time however k_{PS} was kept as small as possible as required by the definition of model I The P_1 value was fitted within $\pm 5\,\%$ in all cases irrespective of the actual experimental error The experimental appearance curves as well as the theoretical best fits of two typical cases are shown in fig 11

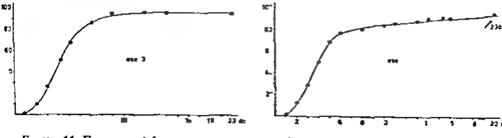

Figure 11 Experimental appearance curves and theoretical best fit on the basis of model Ia in two typical cases

case nr	rate constants (d^{-1})						T	fitting criteria	
	k_{0S}	k_{SP}	k_{PS}	k_{MP}	k_{PM}	$k_{P'M}$		mean dev (%)	max dev (%)
1	—	—	—	14.2	0.18	0.45	4.0	1.2	2.3
2	—	0.55	—	9.45	0.12	0.45	4.0	1.6	3.5
3	—	0.30	—	8.80	0.11	0.49	4.5	1.1	2.4
4	—	1.80	0.10	5.80	0.06	1.20	5.0	1.0	3.5
5	—	1.00	0.02	5.60	0.16	0.60	4.5	0.6	1.4
6	—	0.60	0.01	7.70	0.08	0.40	4.0	0.8	2.2
*7	—	1.10	0.03	4.50	0.08	0.38	4.0	0.6	1.3
*7	—	0.90	0.03	4.70	0.30	0.40	4.0	0.6	1.3
**8	—	0.40	0.03	16.10	0.45	0.30	3.5	0.9	3.3

Table III a

Model I a Best fit solutions in seven normal cases and one case with increased red cell production (**) Rate constants maturation time T and fitting criteria

* *Two examples of possible solutions in case 7 (P_1 value missing)*

case nr	pools (mg)			flows (mg/day)						rel rate of Hb synthesis (%/day)
	S	P	M	J_{0S}	J_{SP}	J_{PS}	J_{MP}	J_{PM}	$J_{P'M}$	
1	—	2.01	45.3	—	—	—	28.5	8.1	20.4	0.76
2	—	2.23	37.0	—	1.23	—	21.1	4.4	16.7	1.00
3	—	1.75	25.7	—	0.53	—	15.4	2.8	12.6	0.82
4	68.8	3.82	17.6	—	6.88	6.88	22.2	1.1	21.1	0.96
5	214.5	4.29	31.6	—	4.29	4.29	24.0	5.1	19.0	1.00
6	249.0	4.04	64.8	—	2.42	2.42	31.1	5.2	25.9	1.15
mean	—	3.02	37.0	—	3.07	—	23.7	4.5	19.3	0.96
*7	161.7	4.41	43.0	—	4.85	4.85	19.8	3.5	16.3	0.64
*7	132.3	4.41	29.6	—	3.97	3.97	20.7	8.9	11.8	0.46
**8	46.7	3.[illegible]	75.2	—	1.40	1.40	56.4	33.8	22.6	1.78

Table III b

Model I a Pool and flow sizes corresponding to the rate constants of table III a (For better understanding the absolute sizes are given as computed on the basis of calculated blood volumes) *Relative rate of haemoglobin synthesis In the mean value cases 7 and 8 are not included*

$\left.\begin{matrix}*\\ **\end{matrix}\right\}$ see table III a

The results obtained in all eight cases are shown in table III. Table III a) contains the rate constants and T which gave the best fit as well as the fitting criteria. As can be seen the mean deviation of the theoretical from the experimental points is equal to or less than the experimental error (1.65 to 2.33 %) indicating that attempts to still better fitting would be meaningless. In table III b) the pool and flow sizes are shown as calculated from the corresponding rate constants in table III a) and from the size of pool P (plasma iron concentration estimated plasma volume). The figures for relative rate of haemoglobin synthesis are independent of blood volume estimates. They show a rather wide variation between the individuals and the mean value is about 15 % higher than the postulated normal value of 0.83 %/day. In view of the many possible experimental errors implicit in this calculation, especially the P_1 value, the model Ia cannot be rejected on the basis of this criterion. In the mean value cases 7 and 8 are not included: case 7 because of lacking experimental data and case 8 because of evidence for increased erythropoiesis (blood letting 2 weeks before the experiment).

The later part of the theoretical disappearance curves (after $t = 1$ day) corresponding to the solutions of table III together with the normal range taken from Pollycove and Mortimer (1961), Chiandussi et al. (1964), Hosain and Finch (1964) and Sharney et al. (1963) are shown in fig. 12. A striking discrepancy can be noticed for all cases except case 8 with increased erythropoiesis. The final slope is about twice the normal value in 4 cases and in the remaining 3 cases (those in which compartment S plays a relatively important role as indicated by an increase in red cell radioactivity beyond the 12[th] day) an additional very slow exponential component can be noticed. There are only two possible explanations for this discrepancy. Either the experimental range observed by different authors (and notably in very good agreement with each other) is subject to some systematic error or model Ia does not represent a physiologically true solution. Concerning a possible systematic error in determining the later part of the disappearance curve one has to consider the influence of slight haemolysis during preparation of the plasma samples. Hosain and Finch (1964) on the basis of dialysis experiments showed that haemoglobin iron actually contributes to the plasma radioactivity measured after the first day after injection. This contribution however was found to be of minor quantitative importance and would according to these authors not be able to change the shape of the disappearance

curve significantly. Thus the conclusion seems difficult to escape that model Ia is not compatible with available experimental data.

In order to corroborate this finding the combinations of rate constants as calculated by Pollycove and Mortimer (1961) in 13 normal individuals were fed into the computer program for model Ia together with varying length of maturation time T and a parabolic distribution of uptake rates. The disappearance curves now fitted perfectly well within the normal range — as it was from this range the rate constants were calculated — but the generated appearance curves differed significantly from the experimental ones irrespective of the length assigned to the maturation time T. The most important difference was found

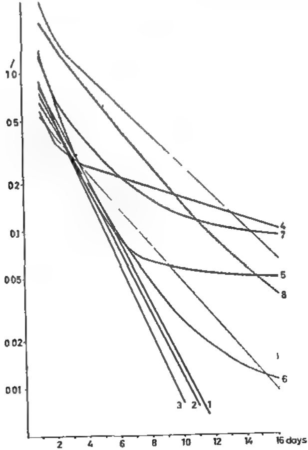

Figure 12 Theoretical disappearance curves after $t=1$ day corresponding to the best fit solutions obtained by fitting the parameters of model I a to the eight experimental appearance curves. The numbers in the figure refer to the respective cases. The shaded area represents the normal range (Pollycove and Mortimer 1961, Chiandussi et al. 1961, Hosain and Finch 1964, Sharney et al. 1963)

in the increment between $t=8$ and $t=14$ days. The experimental values of this increment in the present series as well as those from Pollycove and Mortimer 1961 (fig. 3, p. 755) together with the theoretical values obtained on the basis of model Ia and with rate constants compatible with the observed disappearance curves are shown in fig. 13. The experimental values of the two different series are consistent whereas the corresponding theoretical values are significantly higher ($p<0.001$) if $T=4$ days. If $T=0$ days the probability that there is no difference between theoretical and experimental values is still less than 0.1. Thus, one has to conclude that model Ia is not compatible with all available experimental data.

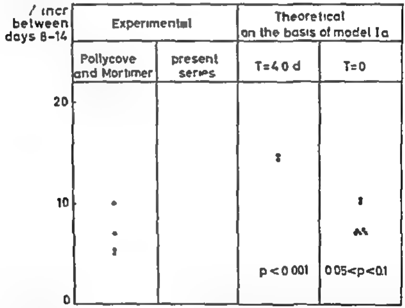

Figure 13. Comparison of the experimentally observed increment in the appearance curve between days 8 and 14 with the corresponding theoretical increment computed on the basis of model Ia and with rate constants compatible with experimental disappearance curves.

curve significantly. Thus the conclusion seems difficult to escape that model Ia is not compatible with available experimental data.

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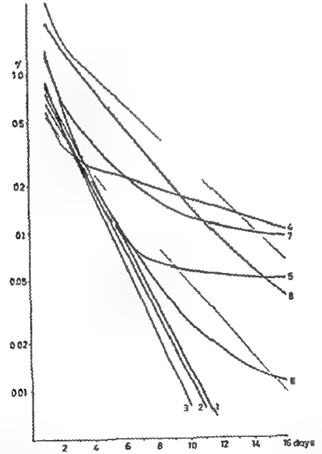

Figure 1? Theoretical disappearance curves after $t=1$ day corresponding to the best fit solutions obtained by fitting the parameters of model Ia to the eight experimental appearance curves. The numbers in the figure refer to the respective cases. The shaded area represents the normal range (Pollycove and Mortimer 1961, Chiandussi et al. 1964, Hosain and Finch 1964, Sharney et al. 1963).

Model Ib takes this concept into account. Its basic structure is shown in fig. 11. Pool M is now incorporated in the red cell precursors, participates in maturation and takes up iron from plasma according to a parabolic distribution of uptake rates as a function of precursor age. Analogously to pool Pr, M is divided into a number of subcompartments m_i, the total number λ being equal to the number of subcompartments pr_i. At each time interval these subcompartments m_i do not only take up iron from P, but a certain portion of their iron content is fed back to plasma according to the same rate constant k_{PM} as in model Ia, and another portion is delivered to the corresponding pr_i according to the rate constant k_{PrM}. These rate constants are assumed to remain unchanged during the whole period of maturation, which means that the chemical nature of the non haem iron is assumed to be identical at all stages of development. Another consequence of this concept is that the rate of haemoglobin synthesis within a single cell is determined by the amount of non haem iron present in this cell at a given moment. If now the rate constants k_{PM} and k_{PrM} and the total maturation time T are relatively small, the non haem iron subcompartments m_i will not be exhausted at the moment of delivery to the circulation. Some assumptions have therefore to be made about the fate of this non haem iron in circulating red cells. According to Faber and Falbe Hansen (1959), Mylhre (1964), Gardner and Nathan (1962) and Najean et al. (1964), the amount of labelled or non labelled non haem iron in circulating red cells is negligible in normal humans except for the first day after injection of radioactive iron, when a considerable fraction of the radioiron present in reticulocytes may be non haem iron. The work of Najean et al. (1964) on the rate of Glycine—C^{14} and Fe^{59} incorporation into newly synthesised haem of reticulocytes suggests that up to 10% of the haemoglobin present in mature erythrocytes is synthesised in the reticulocyte stage, the iron needed for this synthesis being derived mainly from intracellular storage. This notion was incorporated in the present model in the sense that haemoglobin synthesis is allowed to continue from an intracellular non haem iron pool eventually present when a reticulocyte is delivered to the circulation. The time during which this synthesis is allowed to continue is determined by the variable T_R. Feedback to plasma, however, is allowed to occur at the same rate as in the bone marrow during the whole life span of the circulating cells. Iron uptake from plasma is not allowed to occur in circulating cells, as even a very slow uptake would yield too high values in the appearance curve at $t=1$ day.

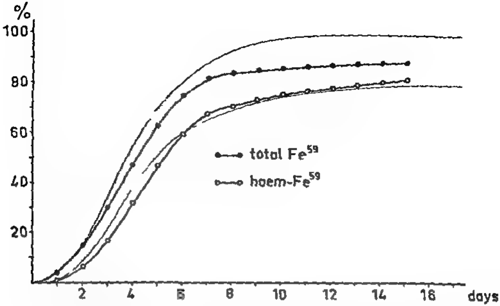

Figure 15 Theoretical appearance curve produced on the basis of model Ib. The shaded area represents the normal range

$\lambda_{OP}=0.4$ $\lambda_{MP}=8.8$ $\lambda_{PM}=0.13$ $\lambda_{P_1M}=0.26$ $T=7.0$ days
$T_R=2.0$ days

As can be seen in fig. 14 pool S is not present in model Ib since the purpose of this chapter was to see whether the rate constants calculated by Pollycove and Mortimer (1961) on the basis of plasma data would be able to generate acceptable appearance curves when pool M is distributed among the red cell precursors and these authors reject the existence of a significant lateral pool S in the normal man. Fig. 15 shows a theoretical appearance curve which was produced on the basis of model Ib and with the mean rate constants of Pollycove and Mortimer. T was 7 days, T_R 2 days and a parabolic distribution of uptake rates from P to M was used. As can be seen, the curve for total radioactivity in the circulating red cells does actually follow a course within the normal range (shaded area), but nevertheless it would not be possible to fit an individual curve, as the increase is too slow after $t=T$. Moreover the figure shows that during the whole 2 weeks period a considerable fraction of the total radioactivity is non haem iron.

In model Ib the rate constants together with the periods T and T_R and the distribution of uptake rates determine not only the behaviour of radioactive iron in a hypothetical experiment but also the amounts of *non labelled* iron present as non haem and haem in erythroblasts, circulating reticulocytes and red cells. Fig. 16 shows the relative amounts

of non labelled non haem and haem iron as a function of maturation stage in a case with the same values assigned to each parameter as in fig 15 The predominance of non haem iron within the bone marrow is very striking In spite of the fact that about 20 % of total haem synthesis would occur in the circulating reticulocyte the total amount of synthesised haemoglobin in bone marrow and peripheral blood would be significantly less than normal (in the present case 0 72 % per day of total circulating haemoglobin mass as compared to the normal rate of 0 83 % as deduced from the plasma data alone) A T value of more than 7 days would of course lead to some reduction of the relative amount of non haem iron present in the reticulocytes when they leave the bone marrow but then the expected curve for total radioactivity in circulating red cells would no longer fit in the normal range It may thus be concluded that the model Ib with the rate constants proposed by Pollycove and Mortimer is not compatible with all available data on the non haem iron content of circulating erythrocytes

Further work on model Ib however revealed that there actually exists a possible solution In the following only that solution will be presented which after several unsuccessful attempts proved to be compatible with experimental radioiron data in plasma and red cells

The following assumptions were necessary in order to get acceptable fits with respect to radioiron data in plasma and red cells

1 The rate constant k_{Prv} is about 1 5 times as high as that proposed by Pollycove

2 The non haem iron reaching the circulation in reticulocytes is

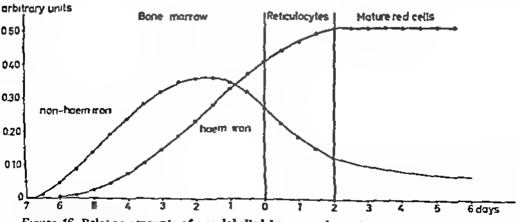

Figure 16 Relative amounts of non labelled haem and non haem iron as a function of precursor age predicted on the basis of model Ib

rapidly cleared from the circulation by a mechanism other than passive diffusion back to plasma

3 The maturation time within the bone marrow is longer than 7 days and the distribution of uptake rates is asymmetrical with a peak at about 3—4 days before the point of delivery to the circulation (cf page 44)

Assumption 2 needs some further comment Crosby (1957) observed that splenectomised patients often have a considerable number of siderocytes in the circulation In experiments where such siderocytic blood was transfused to subjects with intact spleen the siderocytes disappeared very rapidly without any concomitant loss of red cells as measured by the Cr^{51} or the Ashby technique When siderocytic blood was transfused to splenectomised patients however the transfused siderocytes did not disappear from the circulation. Crosby concludes that the non haem iron in siderocytes is removed by a selective mechanism in the spleen whereas the cell as such is left in the circulation This hypothesis was simulated in model Ib by adding a pool *Sp* to which the iron from the non haem iron compartment in reticulocytes is directed with a speed determined by a rate constant k_{SpVr} From this pool the iron is assumed to be fed back to plasma rather rapidly according to a rate constant k_{PSp}

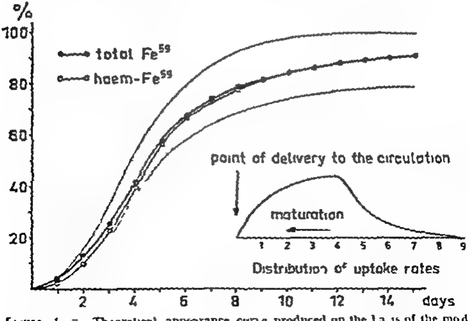

Figure 1 Theoretical appearance curve produced on the basis of the modified model Ib including pool *Sp* The shaded area represents the normal range

k_{P} = 0.3 k_{V} = 8.0 k_{PJ} = 0.15 k_{PV} = 0.40 k_{pV} = 2.0 k_{PSp} = 0.5

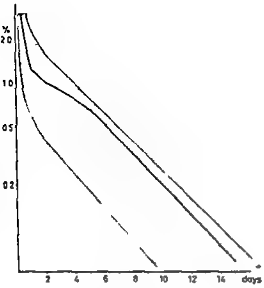

Figure 17 b Later part of theoretical disappearance curve corresponding to the solution of fig 17 a

Fig 17 a) shows the appearance curve of a theoretically possible solution The corresponding rate constants are presented in the figure as well as the distribution of uptake rates within the bone marrow The appearance curve now follows the generally observed pattern and the fraction of non haem radioiron is negligible The corresponding disappearance curve as shown in fig 17 b) is also compatible with experimental data However a certain "hump is quite evident between days 2 and 5 caused by the delayed return of relatively heavy labelled iron from the pool *Sp* According to Hosain (1964) very careful measurements are in fact capable of demonstrating the existence of such a hump Of course there exist several possibilities for explanation and the hypothesis suggested in this section is only one of them The main requirement for a model which should be able to explain a hump in the disappearance curve is some sort of pipeline which provides a delay of radioiron return without concomitant dilution by non labelled iron as is always the case in classical multicompartment systems

Model Ib thus appears to offer a theoretical possibility of a compatible solution where the necessary delay for radioactive iron appearance in circulating red cells is composed of II factors A feedback of non haem iron from maturing red cell precursors to plasma a relatively long total maturation time and a clearance of the non haem iron in reticulocytes by the spleen with consecutive delivery of this iron to plasma This model implies however a rather slow incorporation of the radioactive iron into the haem fraction of red cell precursors The

ratio haem radioiron/total radioiron in bone marrow as a function of time after injection is shown in fig 18 Noyes Hosain and Finch (1964) Myhre (1964), Najean et al (1964) and Pollycove and Maqsood (1962) measured this ratio in man and in animals For comparison the results of these authors are also shown in fig 18 Only the results obtained *in vivo* are depicted in the figure as *in vitro* measurements seem to yield too slow rates of Fe^{59} — incorporation into haem (Myhre 1964) There seems to be fairly good agreement between all these results The lowest figures were found by Pollycove and Maqsood in dogs but even these figures would not be in accordance with the incorporation rate predicted on the basis of model Ib Therefore model Ib is not considered to be acceptable and no attempts were made to fit the rate constants to the individual appearance curves

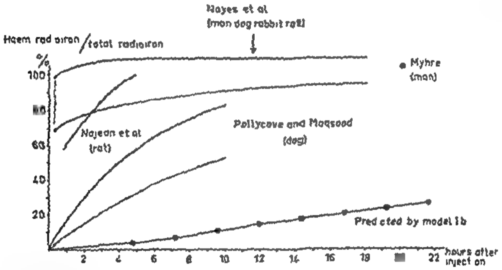

Figure 18 Rate of radioiron incorporation into the haem of bone marrow Experimental data published by different research groups and rate predicted on the basis of model Ib

CHAPTER V

Model IIa

Model I has been defined to represent that special case of the general three compartment model of Garby et al (1963) where the main feedback of iron to plasma was assumed to occur from a labile pool interposed between plasma iron and the iron which is irreversibly fixed in the haem of red cell procursors In chapters III and IV it was shown that this model is not compatible with experimental data whether the labile pool was conceived as a homogeneous and with respect to precursor maturation stationary compartment (model Ia) or with the modification (model Ib) that this pool is incorporated in the maturing red cell precursors

Therefore the other special case of the model of Garby et al (1963) has now to be investigated The exchange with pool M is here negligible by definition and the feedback to plasma is assumed to

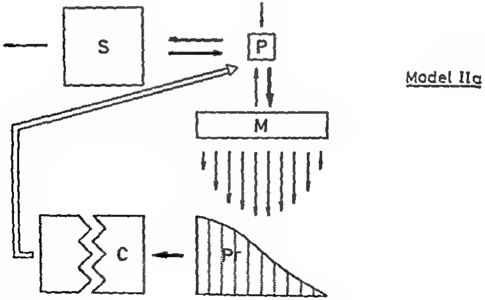

Figure 19 Model IIa

occur from the "lateral" pool S This will be called model II Several modifications of this model will be possible and this chapter is concerned with model IIa as depicted in fig 19 As can be seen the general structure is identical to that of model Ia (fig 2 p 15) but the relative importance of exchange with pools M and S respectively is different as indicated by the thickness of the respective arrows

Quite obviously the same computer program could be used in the fitting procedure for both models Ia and IIa As well the same general rules governed the successive variation of rate constants and maturation time T The range of the rate constant λ_{PS} however exceeded that which had been investigated systematically (fig 8) Extrapolation of the general trend depicted in fig 8 to the range actually used in the investigation of model IIa (0 12 to 0 70) did not in any case yield unexpected results Concerning the rate constant λ_{OS} the influence of which had not been investigated systematically it became evident very soon during the fitting procedure that this constant in model IIa affected almost exclusively the amount of radioiron incorporated in red cells at the end of the experiment ($t>10$ days) Therefore a repetition of the systematic variation of the rate constants was considered to be unnecessary

Disappearance curves (first slope and P_1 value) and appearance curves generated on the basis of model IIa were now fitted to the corresponding experimental data of all eight cases by successive variation of the model parameters λ_{PU} was kept as small as possible and it was practically 0 in all cases except case 1 Table IV a and b shows the best fit solutions Again the mean deviation of the theoretical from the experimental curves is no greater than the experimental error and the calculated rates of haemoglobin synthesis are approximately normal Fig 20 shows the later part of the corresponding disappearance curves (after $t=1$ day) which now fit in well in the normal range in all cases Thus model IIa is compatible with the observed behaviour of radioactive iron in plasma and red cells Najean et al (1964) on the basis of *in vitro* measurements of the rate of Fe^{59} incorporation into the haem of young reticulocytes concluded that the feedback of iron from the intracellular non haem iron pool to plasma would not be important enough to explain the behaviour of radioiron in plasma observed *in vivo* These authors therefore proposed a "mamillary" model similar to model IIa of the present work which should account for the results obtained *in vivo* The physiological counterpart of the lateral pool S cannot be determined by measurements in plasma and red cells only The results of surface measure

case nr	rate constants (d^{-1})						T	fitting criteria	
	k_{0S}	k_{SP}	k_{PS}	k_{MP}	k_{PM}	k_{PM}		mean dev (%)	max dev (%)
1	0.00	2.0	0.30	12.2	0.20	0.85	5.0	1.7	3.8
2	0.06	2.8	0.35	7.2	0.02	0.90	5.0	1.9	3.1
3	0.55	2.5	0.36	6.6	0.00	[illegible]	4.5	1.6	5.2
4	0.01	2.1	0.17	5.5	0.01	1.20	5.0	1.1	3.2
5	0.12	2.2	0.28	4.2	0.00	0.85	4.5	0.6	1.7
6	0.08	2.1	0.25	6.2	0.01	0.52	4.0	0.6	1.8
*7	0.16	1.6	0.12	4.0	0.00	0.44	4.0	0.6	1.7
* 8	0.02	10.0	0.70	6.5	0.00	0.80	3.5	0.9	3.0

Table IV a

Model II a Best fit solutions in seven normal cases and one case with increased red cell production (*) Rate constants maturation time T and fitting criteria

* *Extreme solution of all possible ones maximum rate of haemoglobin synthesis*

case nr	pools (mg)			flows (mg/day)						rel rate of Hb synthesis (% day)
	S	P	M	J_{0S}	J_{SP}	J_{PS}	J_{MP}	J_{PM}	J_{PM}	
1	13.4	2.01	23.3	0.0	4.07	4.07	21.5	4.7	19.8	0.73
2	15.2	2.23	17.5	0.92	6.24	5.32	16.1	0.4	15.7	0.95
3	4.8	1.75	16.6	2.65	4.38	1.73	11.6	0.0	11.6	0.75
4	38.2	3.89	17.4	1.53	8.02	6.49	21.0	0.2	20.8	0.95
5	23.0	4.29	21.2	2.83	9.44	6.61	18.0	0.0	18.0	1.03
6	25.7	4.04	47.2	2.05	8.48	6.43	25.0	0.5	24.5	1.09
mean	20.2	3.07	23.9	1.66	6.76	5.10	19.4	1.0	18.4	0.92
*7	25.2	4.41	40.0	4.04	7.06	3.07	17.6	0.0	17.6	0.69
8	48.6	3.50	28.5	0.98	35.0	34.02	22.8	0.0	22.8	1.30

Table IV b

Model II a Pool and flow sizes corresponding to the rate constants of table IV a and relative rate of haemoglobin synthesis In the mean value cases 7 and 8 are not included

$\left.\begin{matrix}* \\ *\end{matrix}\right\}$ see table IV a

ments by Pollycove and Mortimer (1961) however suggest that this pool is not — in any case not entirely — situated in the liver or the spleen as by one day after injection of the isotope about 15 to 30 % would be present in pool S, and such a heavy load is not compatible with the results mentioned above. It seems more reasonable to assume that pool S represents iron binding proteins with similar binding properties distributed throughout the body so that the maximum amount of radioactive iron present in a single organ would be undetectable by surface measurements. The flow J_{0S} probably represents the sum of those amounts of iron which each day are fixed more or less irreversibly in storage compartments and/or excreted from the body.

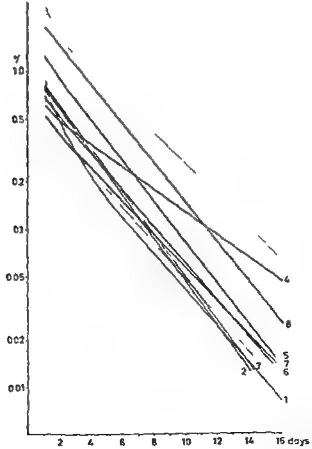

Figure 20. Theoretical disappearance curves after $t=1$ day corresponding to the best fit solutions obtained by fitting the parameters of model II a to the eight experimental appearance curves. The numbers in the figure refer to the respective cases. The shaded area represents the normal range (cf. figure 12).

Model IIa still includes a bone marrow non-haem iron pool of considerable size (approximately 25 mg). The size of this pool M was determined by the shape of the experimental appearance curves, as on the basis of model IIa it was not possible to generate normal appearance curves when M was reduced in size. Since feedback from this pool to plasma is negligible, M simply represents a delay system which should account for the pattern of radioiron appearance in circulating red cells. The interpretation of this system as a bone marrow non-haem iron transit pool is not acceptable in view of the fact that such a system would predict a rather slow rate of incorporation of the radioiron into the haem of red cell precursors. This rate would be approximately twice as rapid as that predicted on the basis of model Ib (fig. 18, p. 38), but still it would not be compatible with the experimental results depicted in the same figure. Other possible explanations of this delay system will be discussed in chapters VI and VII.

CHAPTER VI

Model IIb

In order to explain the necessary delay system encountered in chapter V it may seem reasonable to assume a significantly longer maturation time than that found on the basis of model IIa (4 to 5 days). With a parabolic distribution of uptake rates and a T value of perhaps 10 days however the maximum increment in the appearance curve would be found after the fourth day and this is not compatible with the experimental data. Therefore if the delay system is to be interpreted in terms of prolonged maturation time, an asymmetrical distribution of uptake rates has to be assumed. Model IIb was conceived in order to account for this assumption. Its basic structure is shown in fig. 21. Exchange with pool S is as in model IIa. Pool V however is reduced considerably in size and the maturation time T is extended to 10 or more days. The distribution of uptake rates as a function of precursor age is no longer expressed as a mathematical function; uptake rates are assigned arbitrarily to each subcompartment pr_i. A special computer program was written which allowed the following operations. A number of points along the T axis were chosen and to each point (representing a certain stage of development) a suitable uptake rate in arbitrary units was assigned. The uptake rates for the intermediate stages were then calculated automatically by linear interpolation. The unit was corrected in order to adjust the integral of all uptake rates to 1.0.

Curves generated on the basis of model IIb were now fitted to the experimental data of the eight cases by the following procedure. The rate constants k_{ES}, k_{PS}, k_{P} and k_{-P} were approximately the same as in model IIa and they were found to be determined mainly by the first slope and P value of the disappearance curve and by the appearance curve beyond the 10[th] day after injection. The first part of the appearance curve was fitted by adjusting the distribution of uptake rates along the maturation axis. The rate constant k_{PV} was assigned a more or less arbitrary value. Its order of magnitude

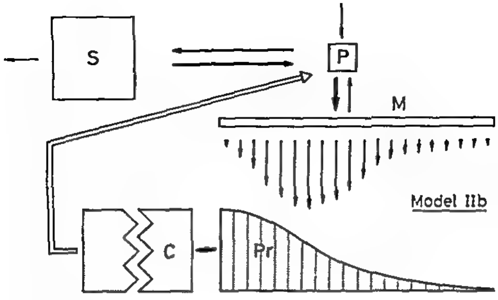

Figure 21 Model IIb

was chosen so that the size of the pool M calculated from J_{MP} and $k_{PU}+k_{PM}$ was approximately equal to the size of the plasma pool P. k_{PM} was kept small as compared to J_{PrM} and it was varied only for the fitting of the P_1 value. From this description of the fitting procedure it is quite clear that the experimental data did not allow a determination of the size of the pool M and the feedback from this pool to plasma in a unique manner. A bone marrow non haem iron pool of the same order of magnitude as the plasma iron pool has practically no influence on the shape of the appearance curve, and a feedback from this pool to plasma would be detected only by very careful measurements in plasma at time $t=0.5$ days to $t=2$ days. Serial measurements during a 24 hours period, however, may be considerably disturbed by physiological diurnal fluctuations, as will be shown in a forthcoming publication (Vuille 1965). At best, maximum and minimum values of these variables might be obtained on the basis of *in vivo* measurements, but this can be achieved only by a randomising mechanism in the fitting procedure. The main purpose of chapter VI, however, is to determine the distribution of iron uptake rates as a function of precursor age which has to be assumed if pool M is of very small size as indicated by the rate of radioiron incorporation into the haem of red cell precursors (fig. 18, p. 38).

case nr	rate constants (d^{-1})						fitting criteria	
	k_{0S}	k_{SP}	k_{PS}	k_{MP}	k_{PM}	k_{PM}	mean dev (%)	max dev (%)
1	0 00	2 3	0 40	11 9	1 2	5 0	1.3	2 8
2	0 06	2 8	0 35	7 2	0 1	9 9	1 6	3 1
3	0 04	1 7	0 28	7 4	0 6	6 7	1 2	2 6
4	0 04	2 1	0 17	5 5	0 1	9 9	1 1	3 3
5	0 09	1 3	0 12	5 1	3 0	7 0	0 5	1 4
6	0 08	2 1	0 25	6 2	0 15	5 5	0 7	1 7
*7	0 16	1 6	0 12	4 0	0 10	3 9	0 6	2 1
**8	0 02	9 0	0 70	7 5	1 00	6 5	0 9	2 2

Table V a

Model II b Best fit solutions in seven normal cases and one case with increased red cell production (**) Rate constants and fitting criteria

* Extreme solution of all possible ones maximum rate of haemoglobin synthesis

case nr	pools (mg)			flows (mg/day)						rel rate of Hb synthesis (%/day)
	S	P	M	J_{0S}	J_{SP}	J_{PS}	J_{MP}	J_{PM}	J_{PM}	
1	11 6	2 01	3 85	0 00	4 62	4 62	23 9	4 6	19 3	0 71
2	15 2	2 23	1 61	0 92	6 24	5 32	16 1	0 2	15 9	0 96
3	9 3	1 75	1 78	0 38	2 98	2 60	13 0	1 1	11 9	0 77
4	38 2	3 82	2 10	1 53	8 02	6 49	21 0	0 2	20 8	0 95
5	26 6	4 29	2 19	2 39	5 58	3 19	21 9	6 6	15 3	0 87
6	25 7	4 04	4 42	2 06	8 48	6 42	25 0	0 7	24 3	1 08
mean	21 1	3 02	2 66	1 21	5 99	[illegible]	20 2	2 2	17 9	0 89
*7	25 2	4 41	4 40	4 04	7 06	3 02	17 6	0 4	17 2	0 67
**8	43 8	3 50	3 51	0 88	31 50	30 66	26 3	3 5	22 8	1 30

Table V b

Model II b Pool and flow sizes corresponding to the rate constants of table V a and relative rate of haemoglobin synthesis In the mean value cases 7 and 8 are not included

$\left.\begin{array}{l}*\\ **\end{array}\right\}$ see table V a

Table V a) and b) summarises the results obtained by fitting the parameters of model IIb to the experimental data of the eight cases. Again a very good fit could be obtained in all cases and the calculated rates of haemoglobin synthesis are close to the predicted normal ones. Fig. 22 shows the distribution of uptake rates which were found to yield the best fit in cases 1 to 7. The shape is strikingly similar for all individuals with a peak value 2.5 to 3.5 days prior to the point of delivery to the circulation and a rather long tail 6 days and more prior to the point of delivery. Fig. 23 shows the range found for these 7 normal individuals together with the curve for case 8 who presented evidence for increased erythropoiesis. The shape of this curve is identical to the other ones but the whole curve is transferred towards the point of delivery by about 1 day. This may be taken as evidence for premature delivery of reticulocytes to the circulation reflecting acute demand for increased haemoglobin production. The solid circles in fig. 23 represent the relative uptake rates as a function of precursor age as calculated on the basis of the "normal part" of the model proposed by Lajtha and Oliver (1960). There seems to be very good agreement except for both extremes of the curve. According to Lajtha and Oliver the last maturation stages would take up considerably more iron but such a model would generate too high figures for red cell radioactivity at $t=1$ day. Actually the figures from Lajtha's model represent relative rate of haemoglobin synthesis rather than rate of iron uptake and it seems conceivable that bone marrow reticulocytes do not take up iron at a high rate but continue haemoglobin synthesis from intracellular non haem iron stores. This concept would be in agreement with the findings of Najean et al. (1964) as discussed p. 33.

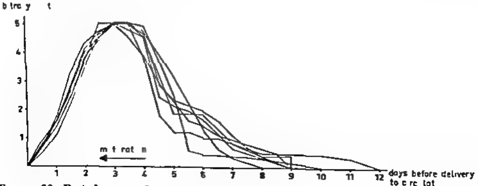

Figure 22. Distribution of uptake rates as a function of precursor age. Results obtained on the basis of model IIb in cases 1—7.

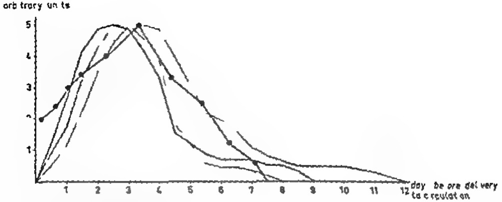

Figure 23 Distribution of uptake rates as a function of precursor age Normal range as in figure 22 (shaded area)
— case 8 (increased red cell production)
— calculated on the basis of the model of Lajtha and Oliver (1960)

The other extreme of the curve the "tail after the 6^{th} or 7^{th} day suggested by the present investigation deserves some further comment Actually, during the fitting procedure the exact length of the tail could not be determined The integral of the tail rather represents that amount of radioactivity which could not be allowed to reach the circulation during the first 6 to 7 days but which had to be incorporated rather rapidly during the following 2 to 5 days It is questionable whether the assumption of a maturation time of up to 12 days is realistic In terms of the model of Lajtha and Oliver this would imply a considerable prolongation of maturation with two to five additional mitotic cycles in the pronormoblast stage There is no experimental evidence to support such an idea and moreover, the large individual variations which one would be forced to assume with respect to the kinetics of erythroblast maturation make this hypothesis still less attractive Therefore other possible mechanisms responsible for the increase in red cell radioactivity between days 7 and 12 which was observed in all cases (5 5—12 0 % mean 9 4 %) will be discussed in chapter VII

CHAPTER VII

Model IIc

The basic structure of model IIc is shown in fig 24 As in model IIb (chapter IV p 32) the pool M is incorporated in the red cell precursors and participates in maturation The subcompartments m_i take up iron from plasma at different rates during the maturation of the cell This distribution of uptake rates is similar to that in model IIb but the "tail beyond the 6th or 7th day is abandonned In order to account for the delayed increase in red cell radioactivity between the 7th and 12th day after injection the model contains two different mechanisms In the first place feedback from pool M to pool P is not restricted *a priori* as was the case in model IIa and to some extent also in model IIb As pool M is considered to be very small such a feedback would affect the plasma disappearance curve mainly during the first 1 or 2 days and would thus be able to account for the intermediate slope in this curve as was observed by Pollycove and Mortimer (1961) and Sharney and coworkers (1963) Wasserman Sharney Gevirtz Schwartz Weintraub Tendler Dumont Dreiling and Witte (1964) presented some experimental evidence that this intermediate slope might at least partially be caused by an exchange of plasma iron with the iron in extravascular fluid According to these authors it seems to be difficult however to draw quantitative conclusions about this point and the experiments of Winchell Pollycove Kusubov Fawaaz and Lawrence (1964) suggest that the contribution of exchange with interstitial fluid to the kinetics of plasma iron is very small and not easily detected The second mechanism incorporated in model IIc is the concept of ineffective erythropoiesis London West Shemin and Rittenberg (1950) measured the excretion of labelled bile pigments in faeces after a single dose of N^{15} labelled glycine They found the bulk of labelled bile pigments being excreted in connection with red cell death about 120 days after administration of the tracer 10 to 20 % of the total amount of labelled bile pigments however was excreted during the very first days and among other possible explanations the authors considered the possibility that this "early peak might be

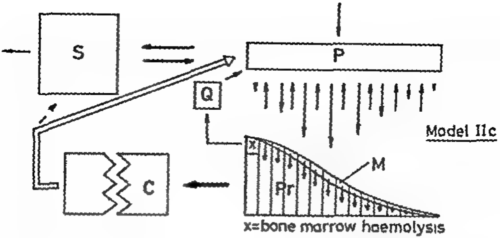

Figure 24 Model IIc

derived from prematurely destroyed erythroblasts or reticulocytes Recently Israels Skandenberg Guyda Zingg and Zipursky (1963) and Yamamoto Skandenberg Zipursky and Israels (1965) were able to show that this early peak is composed of two different fractions The first component of labelled bilirubin was found to appear in plasma within 90 minutes after the administration of labelled glycine and this was believed to be derived from an anabolic pathway The peak of the second component of early labelled bilirubin was observed 3 or 4 days after the administration of labelled glycine simultaneously with the maximum rate of appearance in circulating red cells of labelled haem This observation supports the concept of ineffective erythropoiesis in normal subjects though the figure 10 to 20 % of total haemoglobin synthesis as found by London and coworkers has probably to be reduced somewhat in order to account for the "anabolic component postulated by Yamamoto et al

Incorporation of a mechanism for ineffective erythropoiesis or bone marrow haemolysis into a ferrokinetic model implies the addition of at least three independent and *a priori* unknown parameters In the first place a possible range between 0 and 20 % of total daily haemoglobin production has presumably to be accepted for the total amount of haemoglobin broken down prematurely each day In the second place it is not unimportant for the resulting radioiron curves at which stage of maturation the red cell precursors are assumed to be destroyed Finally a certain delay has to be introduced in order to account for the rate of haem breakdown The addition of these three independent parameters raises the number of possible parameter combinations

in an unreasonable degree. "Manual" adjustment of model parameters in a fitting procedure would not be able to give any information at all about the possible solutions, and even random variation of all parameters would with all probability not lead to any conclusions within reasonable time. We have therefore to look for some experimental observations in the literature which might reduce the degrees of freedom. Premature death of red cell precursors is known to be one of the most important pathogenetic mechanisms in pernicious anaemia. Finch et al. (1956), Pollycove and Mortimer (1961) and Mybre (1964) investigated the behaviour of intravenously injected radioiron in cases of pernicious anaemia. The most important findings were a very high plasma iron turnover (approximately 4 times normal) and a markedly decreased rate of incorporation of the isotope in circulating red cells. Moreover, measurements in plasma after the first day revealed a distinct hump with a peak two or three days after injection. The situation of this peak with respect to the time axis corresponds to that of the small hump in the normal disappearance curve as was observed by Hosain (1964). In chapter IV it was pointed out that the existence of a hump must be taken as evidence for delayed feedback to plasma of iron of relatively high specific activity, which would be the case in a "pipeline" system. Such a possible mechanism was encountered in model Ib, and bone marrow haemolysis would be another explanation for this finding. At least in the case of pernicious anaemia the latter explanation seems to be the only plausible one. The timing of the peak 2 or 3 days after injection of the isotope offers a possibility to evaluate some of the properties of the respective parameters in a model which includes bone marrow haemolysis. By means of a special computer program a hypothetical case of pernicious anaemia was simulated. A definite peak in the disappearance curve around the second or third day could be produced under two alternative conditions. 1. If it was assumed that destruction of red cell precursors takes place at all stages of development or mainly around the middle of development (when iron uptake occurs at a maximum rate), the rate of haem catabolism had to be assumed to be rather slow ($T^{1}/_{2}$ of approximately 1 day). With the assumption of a high rate of haem catabolism, feedback of radioiron to plasma from prematurely destroyed erythroblasts occurred rather immediately and no peak was observed. 2. A peak around the second or third day could be produced in spite of rapid haem catabolism, however, if the bulk of erythroblast destruction was assumed to occur 2 to 3 days after the period of maximum iron uptake, i.e. just at the moment when the cells are ready to leave

the bone marrow. Garby and Noyes (1959) studied the rate of disappearance of Fe^{59} labelled haemoglobin which was injected intravenously and the subsequent appearance in plasma of the liberated Fe^{59}. The maximum rate of Fe^{59} appearance in plasma was observed already after about 1½ hours. This finding of rapid haem catabolism is a strong argument in favour of the latter of the two possibilities discussed above.

Thus we may conclude that the most reasonable model for ineffective erythropoiesis would imply the destruction of a certain number of red cell precursors just prior to leaving the bone marrow and a *more or less immediate return of their iron to plasma. In this case* only one variable is left, namely the fraction of cells that are destroyed at each time interval. It might be argued, of course, that cell death as such is probably a rather slow process and that disturbances in cell metabolism (especially the capability of iron uptake) may be present a long time prior to the final decomposition of the cell. Such a "realistic" model would require a special model for maturation and iron uptake for those cells which are ultimately destinated to die prior to reaching the circulation. We feel, however, that available experimental data would not be sufficient to allow a determination of the parameters in such a model and therefore the simpler concept outlined above is preferred.

In order to get some ideas about the influence on the expected radioiron curves of the rather small amount of ineffective erythropoiesis which can be assumed in normal individuals, the model was applied to case 7, in which the "tail" in the distribution of iron uptake rates had been most pronounced (fig. 22, chapter VI). Fig. 25 shows the

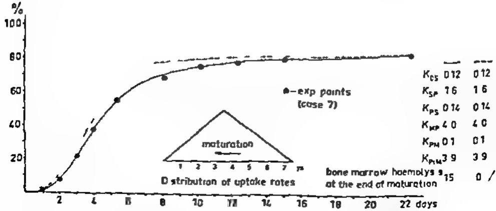

Figure 25. Effect on the theoretical appearance curve of bone marrow haemolysis. Model IIc.

results concerning the appearance curve The dotted line represents the theoretical appearance curve which results when the total maturation time is assumed to be 7.5 days and the rate constants similar to the best fit solution in model IIb The solid line represents the theoretical curve obtained when 15 % ineffective erythropoiesis is assumed As can be seen a good fit is obtained This result suggests that it would be possible to explain the slope of the normal appearance curve on the basis of model IIc but it does not as yet claim that the actual amount of ineffective erythropoiesis can be deduced from these experimental data

Before trying to answer this question we have to look at the other hypothesis put forward in the beginning of this chapter whether a more significant feedback from pool M to plasma would serve as well as an explanation of the observed increase in red cell radioactivity between days 7 and 12 The solid line in fig 26 shows the theoretical appearance curve resulting if about 30 % of the iron reaching pool M from plasma is returned to plasma the size of pool M and all other rate constants being the same as for the best fit solution in model IIb except k_{es} which had to be reduced somewhat in order to fit the last part of the appearance curve Again this possibility cannot be ruled out on the basis of the experimental appearance curve Fig 27 shows the corresponding disappearance curves after $t=1$ day for both solutions It is evident from the figure that a rather important feedback from M to P would account for the second slope between 0.3 and 2 days which was observed by Pollycove and Mortimer (1961) and Sharney et al (1963) whereas the solution with bone

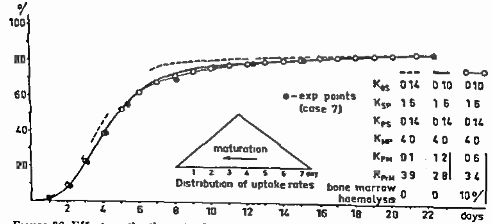

Figure 26 Effect on the theoretical appearance curve of feedback from pool M to P and of the combination feedback $M \rightarrow P$ and bone marrow haemolysis Model IIc.

marrow haemolysis would account for a certain hump at days 2—3. Both features however seem to be somewhat exaggerated in this example and therefore the assumption of a combination of both mechanisms is most close at hand. The open circles in fig. 26 represent the appearance curve of such a combined solution (10 % ineffective erythropoiesis and 15 % feedback from M to P). The corresponding disappearance curve is included in fig. 27. The theoretical P_t values differ widely for the three suggested solutions and therefore this figure may be very important for the choice of the solution(s) in individual cases.

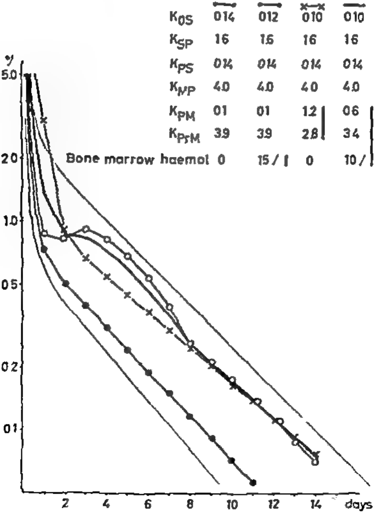

Figure 27. Effect on the theoretical disappearance curve of bone marrow haemolysis and/or feedback from M to P

In all models treated hitherto it was assumed that all iron return from senescent red cells occurs directly to plasma. This assumption has no importance for the determination of rate constants from the experimental radioiron data. When calculating the size of pools and flows, however, one has to have some ideas about this iron return. The possibility of this iron entering pool M directly without passing through plasma can with all probability be rejected because this concept would yield much too high values for haemoglobin synthesis (approximately 5 %/day of total circulating haemoglobin mass). Return to pool S, however, is a much more plausible hypothesis, expecially if this pool now is defined as an integral of iron binding proteins distributed throughout the body. It seems quite reasonable to imagine that within a haem metabolising cell the iron split off is first captured by some iron binding protein which is in exchange with plasma. If in model IIb $J_{SC}=J_{0C}$ and $J_{PC}=0$ the calculated size of the pool S would be approximately 4 times higher than the size given in table Vb (p. 46). The same considerations would be valid also for model IIc. However, as was already pointed out in chapter II (p. 10), the calculated size of the pool S has to be looked upon as a purely mathematical entity, the physiological or chemical counterpart of which cannot be deduced from plasma- and red cell radioiron data alone. Therefore, this calculated size can hardly be used as an argument in favour or against the concept of iron return to S. As in our opinion the pathway from the haem of dying cells via an intracellular labile transport system to plasma is somewhat more plausible than a one directional pathway, iron return to S will be chosen for the further treatment of model IIc. This concept is supported to some extent by the findings of Noyes, Bothwell and Finch (1960) that the turnover of the iron derived from damaged red cells is rather slow in subjects with normal iron stores, whereas this turnover is greatly increased in iron deficient subjects.

To find an acceptable fit on the basis of model IIc would probably be an easy task in all cases. The model contains, however, too many independent parameters, and therefore there is no hope of finding one unique solution for each case on the basis of the actual experimental data. The best thing which can be done is to search for a range of possible solutions.

The following procedure was used in a pilot study in order to explore this problem (Groth, Sandevall, Schneider and Vuille 1965). An automatic curve fitting procedure was developed in which starting values of the parameters could be chosen at random within a wide range. These parameter values were then corrected successively using the heuristic rules concerning the relative influence of the

different parameters on the various regions of the disappearance and the appearance curve which had accumulated during the preceding work. A fit was defined as acceptable if the theoretical curves fell within a range given by the experimental points ± the experimental error. The program was written in FORTRAN IV and run on an IBM 7044. In order to reduce the degrees of freedom, a minimum value for the ratio *Haem radioiron/total radioiron* in bone marrow 5 hours after "injection" was used as additional experimental data (0.6, cf. fig. 18, p. 38), as well as the radioactivity in plasma 2 and 3 days after injection. The experimental data of case 6 (in which the radioactivity in plasma 2 days after injection had been measured; for the activity on day 3 only a probable upper limit — 0.1 % more than on day 2 — was used) were fitted 10 times by this procedure. The maturation time T was unchanged (7.5 days as suggested by Lajtha and Oliver, 1960). The resulting estimates of the rate of effective haemoglobin synthesis showed only moderate variation (range 0.85—0.97 % per day of circulating haemoglobin mass) and were consistent with the predicted normal value. The lowest precision was found in the estimates of the size of the pool S (7.5—17 mg) and the flow J_{0S} (1.8—8.3 mg/d). All other flows and the amount of bone marrow haemolysis (8—12 % of total haemoglobin synthesis) could be determined with rather high precision. The experimental plasma data 1—3 days after injection were found to be of minor importance for the estimate of the rate of effective haemoglobin synthesis. A certain correlation was found between the theoretical value for plasma activity on day 1 and the corresponding estimate of the rate of haemoglobin synthesis, but no such correlation existed for the theoretical plasma activity on days 2 and 3. The latter data were found to be helpful, however, for the determination of the amount of ineffective erythropoiesis.

These preliminary results suggest that an accurate and rather precise determination of the rate of haemoglobin synthesis is possible on the basis of the proposed model and with experimental data that can be obtained with rather low doses of radioactive iron. Before the model can be investigated in further detail, however, additional experimental information, especially concerning the kinetics of erythroblast maturation and the importance of the intracellular non haem iron pool as a precursor of haem iron, is necessary.

General discussion and summary

In extension of the work of Garby et al (1963) the present study was designed to investigate the question how much information can be derived from the red cell appearance curve in individual cases with respect to ferro erythrokinetics In chapter II the reasons were discussed why the original analytical method was abandonned and the method of numerical integration on a digital computer chosen for the production of *theoretical curves and their fitting to experimental* radioiron curves In chapter III model Ia was presented the basic feature of which was the assumption of a significant feedback to plasma from a labile iron pool situated between the plasma compartment and the iron which is irreversibly fixed in the haem of red cell precursors On the basis of this model some results of general importance were obtained concerning the accuracy of the method the influence of the various model parameters on the theoretical radioiron curves and concerning the question of uniqueness of the solutions Fitting of the theoretical appearance curves generated on the basis of model Ia to the experimental appearance curves of 7 healthy individuals yielded approximately normal values for the calculated rate of haemoglobin synthesis The corresponding disappearance curves however were not compatible with the normal range observed by other authors Using rate constants proposed by these authors for the production of theoretical appearance curves the finding of model Ia being incompatible with experimental data could be corroborated In model Ib (chapter IV) the labile pool between plasma and the haem of red cell precursors was incorporated into the maturing cells A compatible solution on the basis of this model could be obtained only if it was assumed that the non haem iron of circulating red cells is rapidly cleared by the spleen and fed back to plasma The predicted ratio *haem radioiron/total radioiron* in the bone marrow as a function of time after injection however was significantly different from the corresponding experimental data published by several research groups Model Ib was rejected on the basis of this finding In chapter V model

IIa was treated the basic feature of which was the assumption of a significant feedback to plasma from a lateral pool On the basis of this model solutions compatible with radioiron data in plasma and red cells could be obtained Still however a delay system between plasma and the haem of red cell precursors had to be assumed which in model IIa was represented by a "transit pool In model IIb (chapter VI) this delay system was interpreted in terms of a much longer precursor maturation time than that which had been used in the preceding models Whereas a parabolic distribution of iron uptake rates as a function of maturation stage had been assumed in models Ia and IIa this distribution was found to be asymmetrical with a rather long "tail for the earliest precursor stages if model IIb had to be compatible with experimental data Except for the "tail mentioned above these results were in very good agreement with the bone marrow model proposed by Lajtha and Oliver (1960) In chapter VII a modification of model IIb was presented as model IIc including a certain amount of feedback to plasma from a small non haem iron pool within the red cell precursors as well as a certain amount of ineffective erythropoiesis (bone marrow haemolysis) By inference from experimental data found in the literature it was concluded that the most reasonable model for bone marrow haemolysis would be represented by the concept of cell death just at the end of maturation in the bone marrow and rather immediate return of the liberated iron to plasma The concept of bone marrow haemolysis would account for a certain hump in the disappearance curve which had been observed by other authors and feedback from a small non haem iron pool would serve as an explanation for the rather unimportant intermediate slope usually found in the disappearance curve 0 5 to 2 0 days after injection Fitting of the parameters of model IIc to the experimental curves of one case demonstrated the compatibility of the model but it was argued that probably several different combinations of parameters would be able to fit the experimental data To test this hypothesis an automatic curve fitting program was written in which starting values of the parameters could be chosen at random within a wide range Fitting the experimental data of one case 10 times by this procedure showed that the rate of haemoglobin synthesis can be estimated with rather high precision by this method whereas the estimates of the size of the lateral pool S showed great variation

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Computer simulation of ferrokinetic models

II Significance of diurnal and day to day variations of plasma iron concentration with respect to ferrokinetics

In 1941 Vahlquist reported significant and consistent differences between morning and evening values of serum iron concentration in healthy adults. Thereafter several investigators (Hemmeler 1944; Hoyer 1944 b; Waldenström 1946; Hamilton, Gubler, Cartwright and Wintrobe 1950; Laurell 1953) have confirmed these findings. According to Hoyer and Hemmeler the fluctuations in serum iron concentration seem to be related rather closely to the rhythm of activity and sleep with a decrease during the day to approximately half the morning value and a consecutive increase during the night. In night workers being adapted to their rhythm the fluctuations of serum iron concentration are generally reversed. The serum iron concentration varies not only during the day. According to Hoyer (1944 a) considerable variations occur also from day to day.

With respect to ferrokinetics these observations are of great importance since the sizes of pools and flows calculated on the basis of a kinetic model are directly related to the measured plasma iron content and are assumed not to vary with respect to time. Generally ferrokinetic studies with radioactive iron are started in the morning with a measurement of the plasma iron turnover and during the following days or weeks measurements of radioactivity in plasma and/or red cells are performed once a day. In the procedures proposed by Pollycove and Mortimer (1961) and Vuille (1965) quite reasonable values were obtained for the most important parameter in ferrokinetics of normal individuals, namely the calculated rate of haemoglobin synthesis, in spite of the fact that fluctuations of the plasma iron concentration were not taken into account. The deviations of the cal-

culated rates of haemoglobin synthesis from the corresponding rates predicted on the assumption of a red cell life span of 120 days and a steady state were significantly less than the deviations which would be expected if the fluctuation in the plasma iron content reflected itself directly on the calculated ferrokinetic parameters. Therefore it seems to be of interest to search for a theoretical explanation of this apparent insensitivity of present ferrokinetic techniques with respect to the diurnal variation of plasma iron content.

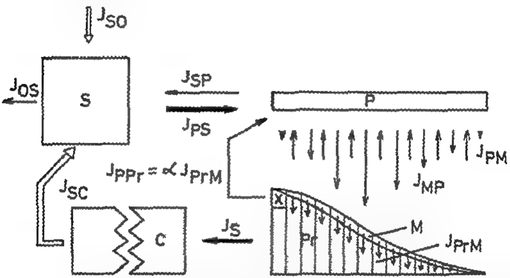

Figure 1 The basic structure of the model used for the present analysis. Iron leaves the plasma in two directions. A certain proportion of the total outflow is directed towards the pool *S* which represents iron loosely fixed to iron binding proteins distributed throughout the body. This pool receives iron also from senescent red cells (J_{SC}) and a relatively small amount from the gut and from slowly exchanging storage compartments (J_{SO}). The most important outflow from this compartment is directed towards the plasma (J_{PS}) whereas J_{OS} ($= J_{SO}$) stands for excretion and more or less irreversible fixation in storage. The major part of the outflow from the plasma is directed towards the bone marrow where the iron is bound in the pool *M* representing exchangeable non haem iron within the red cell precursors. These precursors as symbolised by a set of subcompartments pr_i and m_i take up iron at varying rates during their maturation in the bone marrow. In the figure the maturation procedes from right to left with a total maturation time *T*. The varying length of the arrows between *P* and *M* indicates the distribution of uptake rates as a function of precursor age. At the end of maturation each subcompartment pr_i and m_i adds its accumulated iron to the pool *C* which represents the circulating red cell mass. A certain proportion (bone marrow haemolysis α) however is returned directly to plasma. For the present analysis the precursor part of the model (distribution of uptake rates and maturation time *T*) was unchanged, *T* being assigned the value of 7.5 days. As well the value of α was never changed (5 %) (cf. Vuille 1965).

In a previous publication (Vuille 1965) a method was developed for the simulation of ferrokinetic models by means of numerical integration on a digital computer. In contrast to current "multicompartment analysis" methods the simulation technique allows the introduction of time dependent rate "constants" into the kinetic model. The present communication deals with the results of the following computer experiments. The ferrokinetic events in a hypothetical healthy subject were simulated on the basis of the model depicted in fig. 1 (cp. Vuille 1965) and with diurnal variation of the sizes of different pools and flows. The resulting theoretical radioiron curves in the plasma (disappearance curve) and the red cell (appearance curve) compartments were then taken as representing actually observed radioiron data. In a second step theoretical radioiron curves produced on the basis of the same model but now with constant pool and flow sizes were fitted to these simulated experimental data. The pool and flow sizes which were obtained by the fitting procedure were compared to the corresponding "true" mean values, i.e. those mean values which had been used in producing the simulated experimental data. The motivation for the use of *simulated* instead of real experimental data is that the true sizes of pools and flows are known *a priori* in simulated experiments. They provide thus a basis for the estimation of a possible systematic error brought about if experimental data from non steady state systems are treated in terms of steady state mathematics.

Basic considerations

The simulation of the diurnal variation of the content of unlabelled iron in the different compartments — especially the plasma compartment — was done on the basis of the following considerations. A decrease of plasma iron content during the day may be thought to be caused by two principally different mechanisms. Either the inflow of iron is significantly lower during the day as compared to the night whereas the outflow is constant throughout the 24 hours period, or the outflow is higher during the day and lower during the night while the inflow is constant. The basic relation (1) describes the variation of plasma iron content at any time t as a function of outflow and inflow at the same time t

$$\frac{dP(t)}{dt} = J_i(t) - J_o(t) \tag{1}$$

where $J_i(t)$ = Inflow at time t (mg/d or µg/min)
$J_o(t)$ = Outflow at time t

In this equation $J_i(t)$ and $J_o(t)$ are unknown, whereas $P(t)$, i.e. the integral of dP/dt, or the time course of plasma iron content, has been fairly well characterised by Hoyer (1944 b) and Hamilton et al. (1950). The data of Hamilton et al. suggest that the time course of plasma iron concentration (which with all probability parallels plasma iron content, cf. Bothwell and Mallett 1955) can be described quite closely by a sinus function. Hoyer, however, claims that the period of decrease is significantly longer (approximately 16 hours) than the period of increase during the last part of the night (approximately 8 hours). In order to describe such an asymmetrical time course, a more complicated mathematical function would be needed. For the purpose of the present study, however, the assumption of a symmetrical function was considered to be sufficiently realistic. If the zero point of the time axis is set at 8 a.m. (when plasma iron in general has reached its maximum value), the time course of plasma iron content may be described by

$$P(t) = a \cos 2\pi t + P_m \tag{2}$$

where P_m is the mean plasma iron content and a the amplitude of the variation. Derivation of (2) gives

$$\frac{dP(t)}{dt} = -2\pi\, a \sin 2\pi t \tag{3}$$

Substitution of eq. (1) in eq. (3) gives

$$J_i(t) - J_o(t) = -2\pi\, a \sin 2\pi t \tag{4}$$

The amplitude a is relatively well known in normal individuals and amounts to approximately 1/5 to 1/3 of the mean plasma iron content (Vahlquist 1954, Hoyer 1944 b, Hamilton et al. 1950, Heilmeier 1944). As it is not known with certainty whether a variation of outflow or inflow is responsible for the variation of plasma iron content, both possibilities will be investigated.

a) Outflow assumed to be constant

$$J_i(t) = J_o(\text{constant}) - 2\pi\, a \sin 2\pi t \tag{4 a}$$

b) Inflow assumed to be constant

$$J_o(t) = J_i(\text{constant}) + 2\pi\, a \sin 2\pi t \tag{4 b}$$

Of course, a combination of a) and b) is also imaginable and furthermore it is possible that inflow variation is the real primary mechanism but that there is an additional variation of the outflow as a *consequence* of the variation of the plasma iron content.

In the ferrokinetic model chosen as a basis for this study, both inflow to and outflow from the plasma compartment are composed of two flows, $J_o = J_{SP} + J_{MP}$ and $J_i = J_{PS} + J_{PM}$ (see fig. 1). In discussing the possibility a) (variation of inflow), only J_{PS} will be varied, as the magnitude of the flow J_{PM} is as yet not sufficiently well established. Concerning possibility b), both J_{SP} and J_{MP} will be varied. As the sum of the iron contents in $S+P+M$ is assumed to be constant, either S or M are oscillating inversely to P, depending upon which mechanism is assumed. Only if J_{PM} is varied will the sum $S+P+M$ not be

constant any decrease and increase will be compensated by a corresponding increase and decrease in pool Pr. All mechanisms that have been analysed are summarised in fig. 2 where the time course and the amplitude of the various flow variations are demonstrated schematically as well as the resulting variations of the sizes of the pools S, P, and M.

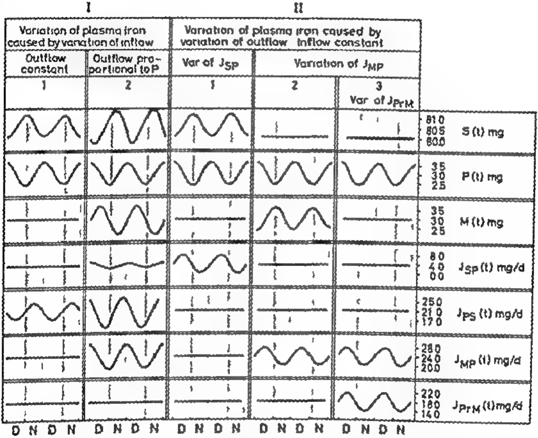

Figure 2. Presentation of five different mechanisms which may be responsible for the diurnal variation of plasma iron content. The time course of those pools and flows of unlabelled iron which are oscillating in one or several of these mechanisms is shown graphically, day and night being symbolised by light and shadowed areas respectively. The mean values as well as the amplitudes can be read at the right margin. The values of the other (non oscillating) flows were: $J_{0S}=J_{S0}=5.0$ mg/d, bone marrow haemolysis $=5\%$ of total haemoglobin synthesised daily, $J_S=J_{SO}=17.1$ mg/d.

Methods

The method of numerical integration of the differential equations describing the changes of radioiron content in the different compartments has been presented in detail in a previous publication (Vuille 1965). In the computer program used there rate constants were introduced as input data. For the present work these "constants" were not introduced a priori but automatically calculated by the following procedure. The time course of the different flows and of the content of unlabelled iron in the various compartments was simulated in the first part of the computer program. Starting values for S, P and M were introduced as input data as well as the mean values of the flows J_{i_m} and a suitable amplitude a_{it} for that flow which was intended to be varied. The simulation was done stepwise by numerical integration with a step length of 0.01 or 0.001 day. The smaller step length was used for the investigation of possible errors in estimating the initial slope of the disappearance curve. At the beginning of the run the time variable t was set at zero and all pool sizes at their starting values. t was then increased by Δt (0.01 or 0.001 day) and the magnitude of the different flows calculated according to the formula

$$J_i = J_{i_m} + a_{it} \sin 2\pi t \tag{5}$$

where J_{i_m} is the mean value of the flow J_i and a_i the amplitude. The flow J_{PP} symbolising bone marrow haemolysis was calculated from the flow J_{P_rM} and a preset fraction α: $J_{PP} = J_{P_rM}\,\alpha$ and $J_S = J_{P_rM} - J_{PP_r}$. From these flows and the preceding pool sizes the actual pool sizes at time t were calculated according to

$$
\begin{aligned}
S_t &= S_{t-\Delta t} + (J_{SP} + J_{SC} + J_{SO} - J_{OS} - J_{PS})\,\Delta t \\
P_t &= P_{t-\Delta t} + (J_{PS} + J_{PM} + J_{PI} - J_{SP} - J_{MP})\,\Delta t \\
M_t &= M_{t-\Delta t} + (J_{MP} - J_{PM} - J_{P_rM})\,\Delta t
\end{aligned}
\tag{6}
$$

The time variable t was then increased by Δt and the calculations repeated in the same order Thus at the end of each time interval the magnitudes of the pools and flows of unlabelled iron were available Hypothetical radioiron experiments could now be started at any desired time t by switch control When the switch was set on rate "constants were calculated at each step from the pool and flow sizes which had been computed in the first part of the program at the same step according to the formula

$$k_j = J_j / X_i \tag{7}$$

(X_i representing the amount of unlabelled iron in compartment i) These rate "constants were then used in the second part of the program for the calculation of the theoretical radioiron curves in compartments P and C This part of the program was identical to the program used for the treatment of model IIc of the previous publication (Vuille 1965) Throughout the present study the precursor part of the model (structure of the pools M and Pr with respect to

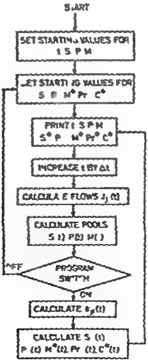

Figure 3 A general flow diagram of the computer program Asterisks indicate that the respective symbols are concerned with radioiron

precursor age) was unchanged with a total maturation time of 7.5 days and a distribution of uptake rates from pool P to M as indicated by the length of the respective arrows in fig. 1.

The fitting procedure (fitting of theoretical radioiron curves produced on the basis of a steady state model to the oscillating curves simulated as described above) was done by manual adjustment of the parameters. A fit was defined as acceptable if the relative deviations of the fitting points from the simulated experimental points were less than 5 % in the later part of the disappearance curve and less than 0.5 % in the initial slope and in the whole appearance curve.

Results and discussion

A. Diurnal variation of plasma iron content

The different mechanisms shown in fig. 2 will be treated in sequence.

1. *Variations of plasma iron content mainly caused by variation of inflow* (J_{PS})

If one assumes that inflow variation is the primary mechanism the question arises whether there is any secondary effect of the plasma iron variation on the outflow. Bothwell and Mallett (1955) measured the plasma iron "turnover" at 10 a.m. and 8 p.m. in each of twenty hospital patients.

In this connection it should be noted that short term tracer experiments in a system which is not in a steady state yield an estimate of *outflow* from that compartment into which the tracer is introduced. Therefore the term "turnover" implying that inflow = outflow does not appear appropriate in this connection. The timing of the experiments by Bothwell and Mallett at 10 a.m. and 8 p.m. indicate that these authors were interested in the question mentioned above, a possible secondary effect on the outflow of plasma iron variation which probably was assumed to be caused primarily by inflow variation though this is not explicitly stated. If the question were to be answered whether outflow variation is the primary mechanism, outflow measurements should be performed in the afternoon and just after midnight, i.e. when the *derivative* of the time course of plasma iron content has reached extreme values (cf. Paterson 1957).

Among the twenty subjects studied by Bothwell and Mallett ten subjects showed a normal disappearance rate of the tracer at one or both occasions whereas this rate was increased above normal at both occasions in the remaining ten subjects (indicating iron deficiency or other disturbancies in iron metabolism). For the "normal" group as a whole the outflow in the evening was not significantly different from the outflow in the morning but considerable individual variations were noticed. At a first glance differences of 20 to 40 percent as were actually observed seem to indicate real variations of outflow as the combined experimental error of radioactivity assay and plasma iron determination should not exceed 5 to 10 %. Very recently similar

results have been reported by Lockner (1964). Also in this material there was no significant difference between morning and evening values for the control group as a whole but individual variations of the same order of magnitude as in Bothwell and Malletts material were observed. The absence of a definite trend in both materials suggests that random factors other than the pure experimental error might affect the results of such studies.

In order to analyse this question the following computer experiments were performed. The "true" events concerning the flows and pools of non labelled iron in a healthy subject were simulated assuming the following mean values (for interpretation of symbols cf. fig. 1):

$S =$ [illegible] mg, $P = 3.0$ mg, $M = 6.0$ mg, $J_{0S} =$ [illegible] mg/d, $J_{PS} = 24.0$ mg/d,
$J_{SP} = 4.0$ mg/d, $J_{MP} = 24.0$ mg/d, $J_{PM} = 2.0$ mg/d, $J_{PEM} = 22.0$ mg/d,
bone marrow haemolysis $= 5\,\%$, $J_{EL} = 20.9$ mg/d.

The variation of plasma iron content was assumed to be caused by variation of the flow J_{PS} with an amplitude $a_{PS} = 6.28$, leading to an amplitude of plasma iron content of ± 1.0 mg. All other flows were assumed to remain constant throughout the 24 hours period. The time course of plasma iron content in this experiment is shown in fig. 4. The arrows indicate the occasions at which the program switch was set on in order to simulate the beginning of a radioiron experiment. The theoretical rate of disappearance of the tracer from the plasma was recorded during the following three hours, then the switch was reset until the time for the next simulated tracer experiment. The

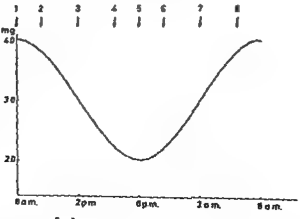

Figure 4. Time course of plasma iron content in the analysis of the effect on outflow measurements. The numbered arrows indicate the occasions at which hypothetical radioiron experiments are started.

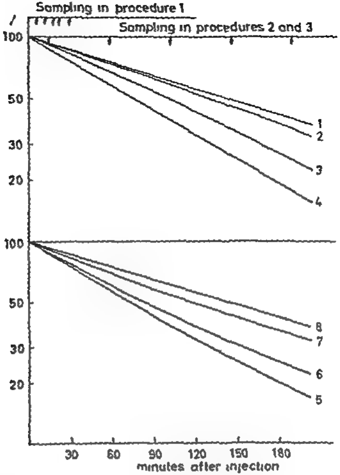

Figure 5 The rate of disappearance of the tracer in a system where the outflow is constant The numbers refer to the occasions at which radioiron experiments are started (cf fig 4) The arrows at the upper limit indicate different sampling procedures

theoretical rates of disappearance of the tracer during the first three hours are shown in fig 5 The numbers in the figure correspond to the numbers of the arrows in fig 4 As can be seen the rate of disappearance increases gradually as the plasma iron content decreases and vice versa For some of these curves a deviation from a single exponential slope can be noticed already during the first three hours but these deviations would hardly be detectable in real experiments (cf Garby Schneider Sundquist and Vuille 1963) From these disappearance rates the outflow was calculated in three different ways corresponding to three different procedures in real experiments

Procedure 1 The disappearance of the tracer is assumed to be measured only during the first 30 minutes after injection five samples being taken at 6 12 17 and 30 minutes respectively and plasma iron content is assumed to be measured at the time of injection

Procedure 2 Five samples for radioactivity measurements are assumed to be taken at 14 58 100 144 and 187 minutes after injection Measurement of plasma iron content is assumed to be done at the moment of injection of the tracer

Procedure 3 Measurements of radioactivity are assumed to be performed in the same order as in procedure 2 but the estimate of plasma iron content is assumed to be based on the arithmetic mean value of five samples taken at the same occasions as the samples for radioactivity measurement

In order to simulate the experimental error of the measurement of radioactivity in a single sample the theoretical values were increased by 1 % for the first sample decreased by 1 % for the second sample and so on (Reversal of the distribution of the + and − signs among the consecutive samples did not significantly alter the results presented below) Determinations of plasma iron content are assumed to be done without any experimental error

From these simulated experimental data the rate of disappearance of the tracer was calculated by the method of least squares according to Snedecor (1957) The product *Rate of disappearance* x *plasma iron content* was taken to represent the actually calculated outflow of iron from the plasma in a subject where this outflow is perfectly constant throughout the day The results are shown in table I The third column in the table contains the calculated rate of disappearance at each of the eight occasions shown in fig 4 The standard error of this estimate is shown in column 4 and the coefficient of variation in column 5 Column 6 contains the calculated outflow of iron in mg/d and column 7 the percent deviation of this estimate from the true value ($J_{SP}+J_{XP}=28.0$ mg/d) From these results the following conclusions can be drawn

1 Experimental procedures 1 and 3 would yield rather accurate estimates of the outflow irrespective of the occasion at which the experiments is started (column 7)

2 The precision of this estimate (coefficient of variation of the rate of disappearance column 5) however would be quite bad for procedure 1 whereas procedure 3 appears to be rather good also in this respect

Exp procedure	Start of exp (fig. 4)	Initial slope (d^{-1})	± s e	Coeff of var %	Calculated outflow (mg/d)	Diff to "true" outflow (%)
	1	6.92	1.02	14.8	27.7	−1.1
	2	7.36	1.02	13.8	28.0	0.0
	3	9.51	1.01	10.6	28.5	+1.7
1	4	12.98	1.01	7.8	28.4	+1.5
	5	13.94	1.03	7.4	27.9	−0.4
	6	12.42	1.06	8.6	27.2	−2.8
	7	9.03	1.04	11.5	27.1	−3.1
	8	7.15	1.03	14.3	27.4	−2.1
	1	7.05	0.12	1.8	28.2	+0.6
	2	7.91	0.12	1.5	30.1	+7.5
	3	10.64	0.17	1.6	31.9	+13.9
2	4	13.35	0.15	1.1	29.2	+4.4
	5	12.85	0.35	2.7	25.7	−8.2
	6	10.79	0.40	3.7	23.7	−15.5
	7	8.00	0.27	3.4	24.0	−14.2
	8	6.90	0.16	2.4	26.3	−6.0
	1	7.05	0.12	1.8	27.3	−2.6
	2	7.91	0.12	1.5	27.4	−2.2
	3	10.64	0.17	1.6	27.5	−1.7
3	4	13.35	0.15	1.1	27.4	−2.2
	5	12.85	0.35	2.7	27.3	−2.3
	6	10.79	0.40	3.7	27.4	−2.2
	7	8.00	0.27	3.4	27.3	−2.5
	8	6.90	0.16	2.4	27.3	−2.6

Table 1 The error of the estimate of *outflow from plasma* brought about by fluctuations of plasma iron content, in the case of constant outflow. Dependence of this error on the experimental procedure

3 With procedure 2 in spite of a relatively high experimental precision the accuracy of the estimate of the outflow is quite low as by this method a "mean" rate of disappearance is computed during a rather long time interval without taking into account the considerable variation of plasma iron content which takes place simultaneously

The systematic slight underestimation of the outflow by procedure 3 (column 7) is due to the fact that in the model used for the present analysis the feedback from M to P (U_{PM}) occurs very rapidly thus leading to a systematic deviation of the estimate of the initial slope towards lower values

corresponds to a "compromise" between procedures 1 and 2 mentioned above and both the accuracy and the precision of the method can thus be expected to be somewhere in between these two extremes In the light of these considerations the possibility cannot be ruled out that the individual diurnal variations of outflow which were observed by these authors are — at least to some extent — artefacts brought about by the experimental procedure If for instance outflow is measured in a particular individual at occasions 2 and 6 respectively (fig 4) by the experimental procedure 2 a "false" difference between these two measurements of 23 % would be observed

Paterson (1967) measured the rate of Fe^{59} disappearance by day and by night in three healthy subjects He found a significantly higher rate of disappearance by day when the serum iron concentration was falling as compared to the night when the serum iron concentration was rising Estimates of outflow however were based on radioactivity measurements during ½ hours and a figure for plasma iron content derived from a single measurement at the time of injection Thus these experiments correspond approximately to the starting points 3 and 7 (fig 4) and the assumed experimental procedure 7 of table II As can be seen a false difference of 28 % would be observed in the case of the present example Differences of the same order of magnitude (20–50 %) were found by Paterson and therefore his conclusion that outflow variation is the only cause of the diurnal fluctuation of plasma iron appears to be somewhat hazardous

1 1 *Outflow constant*

In view of the results presented in the preceding section the assumption of constant outflow cannot be rejected on the basis of present day knowlegde A complete iron kinetic experiment on the basis of the assumed mechanism I 1 (fig 2) was now simulated the starting point being chosen at the peak of plasma iron content The radioiron curves in plasma and red cells during the first two weeks after injection of the isotope which should be expected in this case are shown in fig 6 As can be seen from the figure the oscillations of plasma iron content should reflect themselves in plasma radio activity throughout the study The amplitude of these oscillations is however approximately equal to the experimental error in this region (cp Pollycove and Mortimer 1961) and may therefore not easily be detected in reality No oscillations can be found in the red cell radio iron curve in this curve the increment at any time is in itself an inte gral of processes occurring during the rather long period of precursor

Exp procedure	Start of exp (fig 4)	Initial slope (d^{-1})	± s e	Coeff of var %	Calculated outflow (mg/d)	Diff to "true" outflow (%)
	1	6.92	1.02	14.8	27.7	−1.1
	2	7.36	1.02	13.8	28.0	0.0
	3	9.51	1.01	10.6	28.5	+1.7
1	4	12.98	1.01	7.8	28.4	+1.5
	5	13.94	1.03	7.4	27.9	−0.4
	6	12.42	1.06	8.6	27.2	−2.8
	7	9.03	1.04	11.5	27.1	−3.1
	8	7.19	1.03	14.3	27.4	−2.1
	1	7.05	0.12	1.8	28.2	+0.6
	2	7.91	0.12	1.5	30.1	+7.5
	3	10.64	0.17	1.6	31.9	+13.9
2	4	13.35	0.15	1.1	29.2	+4.4
	5	12.85	0.35	2.7	25.7	−8.2
	6	10.79	0.40	3.7	23.7	−15.5
	7	8.00	0.27	3.4	24.0	−14.2
	8	6.90	0.16	2.4	26.3	−6.0
	1	7.05	0.12	1.8	27.3	−2.6
	2	7.91	0.12	1.5	27.4	−2.2
	3	10.64	0.17	1.6	27.5	−1.7
3	4	13.35	0.15	1.1	27.4	−2.2
	5	12.85	0.35	2.7	27.3	−2.3
	6	10.79	0.40	3.7	27.4	−2.2
	7	8.00	0.27	3.4	27.3	−2.5
	8	6.90	0.16	2.4	27.3	−2.6

Table I **The error of the estimate of outflow from plasma brought about by fluctuations of plasma iron content in the case of constant outflow Dependence of this error on the experimental procedure**

3 With procedure 2 in spite of a relatively high experimental precision the accuracy of the estimate of the outflow is quite low as by this method a "mean" *rate of disappearance is computed during a rather long time interval without taking into account the considerable* variation of plasma iron content which takes place simultaneously

The systematic slight underestimation of the outflow by procedure 3 (column 7) is due to the fact that in the model used for the present analysis the feedback from V to P (J_{PV}) occurs very rapidly thus leading to a systematic deviation of the estimate of the initial slope towards lower values

The experimental procedure chosen by Bothwell and Mallett probably

The initial slope (during the first 30 minutes after injection) as well as the points on the disappearance and the appearance curves at 1 2 3 days and so on after injection were now taken to represent the experimental data to which the parameters of a ferrokinetic model in a perfect steady state were to be fitted The solid line in fig 8 represents the best fit solution The corresponding pool and flow sizes as compared to the real mean values are shown in table II It is evident from these results that the size of the pool M would be underestimated to some degree but no error would result in the estimation of the flows and especially the calculated rate of haemoglobin synthesis would be perfectly valid in spite of the fact that the actual estimate of plasma iron content which is a basic figure for the calculation of all other pool and flow sizes represents a maximum rather than a mean value The first part (up to $t=1$ day) of the plasma radioiron curve (simulated and fitted) is plotted in a larger scale in the same figure in order to demonstrate the difficulties which may be encountered in attempts to fit this part of the curve by a multiexponential curve The open circles represent experimental points that would be obtained if the radioactivity in plasma were expressed as *cpm/µg* iron instead of the usual *cpm/ml plasma* These points would correspond approximately to the curve which is obtained by fitting all other experimental data which means that the error in estimating the size of the pool M cannot be avoided by correcting the plasma activity as mentioned above

Assumed mechanism (cf fig 2)	pools (mg)			flows (mg/d)							
	S	P	M	[illegible]	J_{PS}	J_{SP}	J_{MP}	J_{PM}	J_{PrM}	bone marrow haemol.	J_5
I 1	80.6	36.4	2.0	5.0	21.1	4.0	24.0	6.0	18.0	[illegible]	[illegible]
2	80.6	36.4	1.5	5.0	21.1	4.0	28.0	10.0	18.0	[illegible]	[illegible]
II 1	79.4	36.4	3.0	5.0	23.1	6.0	22.0	4.0	18.0	[illegible]	[illegible]
2	80.0	36.4	2.0	5.0	20.6	3.5	24.5	6.5	18.0	[illegible]	[illegible]
3	80.0	36.4	2.0	5.0	20.6	3.5	24.5	6.5	18.0	[illegible]	[illegible]
"true" mean values	80.6	3.0	3.0	5.0	21.1	4.0	24.0	6.0	18.0	[illegible]	[illegible]

Table II Sizes of pools and flows as estimated by fitting theoretical curves produced on the basis of a steady state model to simulated experimental data produced on the basis of different assumptions concerning the mechanisms responsible for the diurnal variation of plasma iron content.

A similar experiment was performed with the modification that the simulation of the radioiron experiment was started at a time when plasma iron content had reached a *mean* value (3 0 mg) By fitting the curve thus obtained by the procedure described above the same values for all flows were obtained the estimate of the pool M, however was now accurate (3 0 mg)

1 2 Outflow directly proportional to plasma iron content

In the series of Bothwell and Mallett (1955) ten patients showed an increased disappearance rate of the tracer from the plasma both in the morning and in the evening In this group there was a significant trend of outflow being lower when plasma iron concentration was low and vice versa It seems quite conceivable that plasma iron concentration may become the limiting factor for the rate of iron removal in iron deficient subjects This would tally with the observations of Katz and Jandl (1964) and Morgan and Laurell (1963) that the rate of iron uptake by reticulocytes *in vitro* is dependent upon the concentration of iron in the surrounding plasma Assuming proportionality between outflow and plasma iron content such a mechanism may be described mathematically as follows

$$\frac{dP(t)}{dt} = J_i(t) - J_o(t) \qquad (8) = (1)$$

The outflow $J_o(t)$ is now at any time t proportional to $P(t)$

$$J_o(t) = k\ P(t) \qquad (9)$$

As before $P(t)$ is described by

$$P(t) = a \cos 2\pi t + P_m \qquad (10) = (2)$$

and

$$\frac{dP(t)}{dt} = J_i(t) - k\ P(t) = -2\pi a \sin 2\pi t \qquad (11)$$

$$\begin{aligned} J_i(t) &= k\ P(t) - 2\pi a \sin 2\pi t \\ &= k\ (a \cos 2\pi t + P_m) - 2\pi a \sin 2\pi t \end{aligned} \qquad (12)$$

Thus for given values of P_m, a, and k the time course of the inflow is determined. This mechanism was simulated as well (see fig. 2). Both outflows from plasma ($J_{SP}+J_{MP}$) were varied proportionately to plasma iron content. The resulting radioiron curves resembled closely those shown for mechanism I.1. Again the initial slope of the disappearance curve and the points on the disappearance and the appearance curves at consecutive days were taken as representing experimental data. Fitting of the parameters of a steady state model to these data yielded the figures for the sizes of pools and flows shown in the respective row of table II. As can be seen J_{MP} and J_{PM} would be overestimated in this case, whereas the estimate of the size of the pool M would be too low. The most important result however is the fact that the rate of haemoglobin synthesis as estimated by the flow J_S would correspond exactly to the true value.

II. Variation of plasma iron content assumed to be caused by variations of outflow

As mentioned earlier, the results of Bothwell and Mallett (1955) and of Lockner (1964) are not able to answer the question whether variations of outflow from plasma are the cause of the diurnal variations of plasma iron content in normal subjects. The results in iron deficient subjects suggest, however, that this cannot be the case for this group, since outflow variations cannot at the same time be cause *and* effect of variations of plasma iron content. Because the evidence is not as clear for normal subjects (cf. Paterson 1957), this hypothesis will be investigated as well.

II.1. Variation of J_{SP}

If this is to be looked upon as the only cause of the diurnal rhythm of plasma iron (as has been proposed by Schafer 1964), the flow J_{SP} oscillates according to a sinus function with a positive coefficient (higher values during the day, lower during the night). With such a mechanism, identical experiments were performed as described in the preceding sections. The results obtained by fitting the parameters of a steady state model to the theoretically produced "true" curves are included in table II. Minor errors would result in the estimate of some flows, but the estimate of the rate of haemoglobin synthesis would be accurate.

II 2 Variation of J_{VP}

The result of fitting the parameters of a steady state model to the "true" experimental data in a case where the fluctuations of plasma iron content are assumed to be caused by variations of the flow J_{VP} are also shown in table II. Again the rate of haemoglobin synthesis (J_S) calculated in this way would correspond exactly to the true value.

II 3 Variation of J_{VP} and $J_{P,V}$

Here it is assumed that a significant diurnal variation of the rate of haemoglobin synthesis is the primary mechanism. Since the pool V is assumed to be of rather small size (Vuille 1965) such a variation would probably give rise to a more or less parallel variation of the flow J_{VP}. This combined variation of $J_{P,V}$ and J_{VP} as shown in fig 2 was simulated as well. As can be seen from fig 2 the size of the pool V would be invariant with time since in this example both the amplitude and the time course of inflow and outflow to and from this compartment are identical. The corresponding radioiron curves were practically identical to those obtained in example II 2 except the part between $t=0.5$ and 1.0 day. Since this part is not used in the fitting procedure the calculated pool and flow sizes are identical to those of example II 2.

B *Day to day variations*

A basic assumption for the results presented in the preceding sections was a single sinus or cosinus rhythm of plasma iron content implying identical values at the same hour each day. This assumption is certainly an oversimplification. According to Hoyer (1944 a) "the variations at repeated determinations on the individual normal subject are of about the same magnitude as at single determinations on several different (subjects)". The minimum and maximum values obtained by this author from 26 serial determinations in individual subjects differed approximately by a factor 2. As current ferrokinetic techniques include measurements during an interval of one to three weeks the possible influence of such variations has to be considered as well. In a computer experiment similar to those described in the foregoing sections a rhythm of five days was superimposed on the diurnal rhythm. In reality variations from day to day cannot be described by any simple function but for the results emanating from this experiment

the exact time course of day to day variations is of no importance Mechanism I 1 of fig 2 i e a variation of flow J_{PS} was accepted as the direct cause of plasma iron variation — both diurnal and day to day

$$J_{PS}=J_{PS_m}-a_{PS}\sin 2\pi t+b_{PS}\sin(2\pi t/5)$$

J_{PS_m} was 21.1 mg/d a_{PS} was −6.28 and b_{PS} was −1.26 leading to maximum and minimum morning values of plasma iron content of 5.0 and 3.2 mg respectively and a lowest evening value of 1.0 mg The size of the pool S varied inversely to P All other pool and flow sizes were constant with mean values as shown in fig 2 In order to make the possible error most pronounced the simulated radioiron experiment was started when plasma iron content was at its extreme value of 5.0 mg

The resulting radioiron curves as well as the time course of plasma iron content in this example are shown in fig 7 The broken line shows the best fit to the initial slope and the consecutive morning values in the disappearance curve The daily haemoglobin synthesis computed in this way was found to be 5.6% higher than the true value For the appearance curve corresponding to the parameters of this best fit solution a slight but systematic deviation from the "experimental" points can be noticed (broken line) The solid lines (both appearance and disappearance curves) represent the best fit if the initial slope of the disappearance curve and the appearance curve are taken as experimental data The open circles in the disappearance curve represent the radioactivity in plasma expressed as *cpm/µg iron* instead of the usual *cpm/ml plasma* It can be seen that this correction would yield a disappearance curve which — at least from the second day on when tracer equilibrium is established — is perfectly compatible with the corresponding appearance curve Moreover the estimate of the rate of haemoglobin synthesis would be accurate (J_S=17.1 mg/d)

The deviations in plasma radioactivity from an ideal line after t=2 days which may be expected as a result of variations of plasma iron content are approximately within the range of the experimental error Therefore a correction may seem to be unnecessary If the experiment is started at an occasion when plasma iron content has reached an extreme value however a systematic error may be superimposed on the random experimental error and therefore it seems wiser to correct the radioactivity in plasma for the actual content of iron (*cpm/µg* as percent of *cpm/µg* at the time of injection) Prior to the establishment of the last slope (t=2 days in the present system)

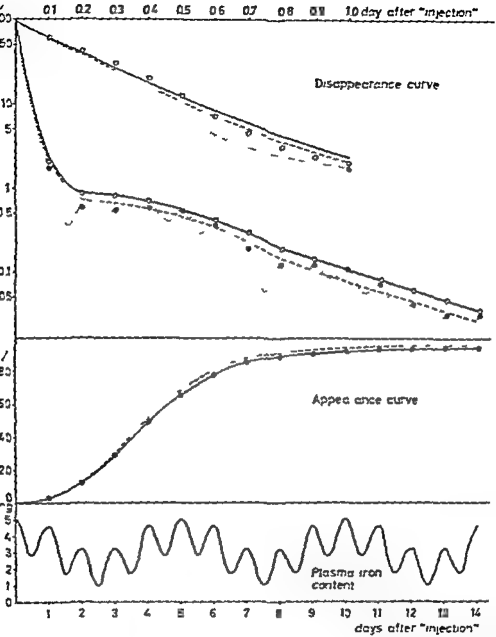

Figure 7 Radioiron curves in day-to-day and diurnal variation of plasma iron content.

Expected radioiron curves (for the appearance curve coinciding with ——)

Curves fitted to the observed morning values in plasma (*cpm/ml* ●)

—— Curves fitted to the observed appearance curve and the morning values in plasma expressed as *cpm/μg* iron (O)

The first part of the disappearance curve (up to $t=1$ day) is shown separately in a larger scale.

The time course of plasma iron content in this example is also shown

this correction appears to be insufficient since it was not possible to fit the point at $t=1$ day together with all other experimental data Furthermore this experiment reinforces the impression that the appearance curve in contrast to the disappearance curve is to be looked upon as "safe experimental data with respect to the non steady state of the exchangeable iron

Conclusions

The results presented in this study give a theoretical justification for the approach of measuring the rate of haemoglobin synthesis by ferrokinetic techniques which neglect the diurnal and day to day variations of plasma iron content. Whatever the mechanism behind the diurnal variations, the calculated rate of haemoglobin synthesis appears to be accurate provided that an appropriate choice of experimental data is made. The same statement is valid concerning the variations from day to day if this variation is *not* caused by fluctuations in the erythropoietic activity of the bone marrow. Variations of the actual rate of haemoglobin synthesis with a high amplitude and a rather low frequency would *a priori* exclude the determination of an accurate mean value by any method which includes measurements of relatively short duration (initial slope of plasma disappearance).

The estimate of other ferrokinetic parameters may be subject to significant error. Especially the size of the pool M in the present model and its hypothetical feedback to plasma may not easily be determined from radioiron data in plasma and red cells. The same consideration is valid also for other hypothetical exchangeable pools of small size that have been postulated (Sharney et al. 1963, Wasserman et al. 1964). The investigation of kinetic models including such pools would require a detailed interpretation of the plasma radioiron curve during the first 48 hours after injection. This part of the curve, however, may be affected considerably by the diurnal variations of plasma iron content, making an accurate interpretation in terms of mean sizes of pools and flows more or less impossible. Expression of radioactivity in plasma as *cpm/µg* iron instead of *cpm/ml* plasma would probably make a fit of theoretical curves to experimental data easier, but nevertheless the results derived from this part of the disappearance curve would have to be interpreted with caution.

The theoretically best experimental procedure that should be used in order to get an accurate estimate of the rate of haemoglobin synthesis deserves some further comment. The results presented in table II are based on the assumption that measurements are performed

only once a day and each day at the same time. Also the determination of the initial slope in the disappearance curve is assumed to be performed during a very short time interval only. The last mentioned procedure cannot be recommended for practical purposes because of the considerable loss of experimental precision (see table I). An estimate of the initial slope based on measurements during 2 hours after injection would probably be the best compromise between the desired experimental precision on one hand and the accuracy of the estimate on the other hand. In this case the accuracy can be increased by determining a mean value of plasma iron content during the same period. Moreover, the experiments should be started at a time when the plasma iron level is reaching an extreme value, as the differences per unit time are smallest at these occasions. The results presented in this study furthermore recommend the use of the appearance curve as "safe" experimental data, because this curve represents an integral of processes occurring during a rather long time interval, thereby "averaging" fluctuations of higher frequency. A more detailed analysis concerning the experimental data which have to be looked upon as important and reliable, and also concerning a possible automatic fitting procedure, will be presented in a forthcoming publication (Groth, Sandevall, Schneider and Vuille 1965).

All mechanisms that have been studied in this work predict diurnal oscillations of plasma radioactivity throughout a 2 weeks experiment. So far, no experimental observations corroborating this prediction have been reported. This lack of experimental evidence is probably due to insufficient experimental precision. The only possible mechanism which would *not* give rise to such oscillations of plasma radioactivity would imply a) the diurnal variation of a flow to plasma from a non exchangeable compartment (for instance iron return from senescent red cells directly to plasma in a one directional way) *and* b) variation of outflow directly proportional to the time course of plasma iron content. The latter requirement does not appear to be fullfilled in normal individuals as indicated by the results presented by Bothwell and Mallett (1955) and Lockner (1964).

This work was not primarily concerned with the biologically interesting question of the possible causes of the variation of plasma iron content. According to Schafer (1964), the activity of the *RES* seems to be the most important factor, since blockage of this system by repeated injections of colloidal silver is able to suppress the spontaneous diurnal rhythm in animals. This would suggest that variations of J_{PS} or J_{SP} or both (in the model used for the present study) are

responsible for the variations of plasma iron Schäfer believes that it is mainly a question of the outflow from the plasma (J_{SP}), since he observed an increase of iron content in the *RES* when serum iron was decreasing This observation can of course be explained as well by the assumption of a decreased flow from the *RES* to the plasma (J_{PS}) Laurell (1953) reported a concomitant diurnal variation of serum bilirubin concentration and concluded from these results that the rhythm of both serum iron and serum bilirubin are due to the same mechanism a diurnal variation of the rate of haem breakdown This would correspond to the mechanism I 1 of this study (variation of J_{PS}) Serial outflow measurements at times of most rapid changes of serum iron concentration (in the afternoon and after midnight) with accurate methods should be able to yield additional information concerning this question

Summary

Ferrokinetic techniques for the determination of the sizes of iron pools and flows have hitherto neglected the considerable diurnal and day to day variations of plasma iron content although the estimate of plasma iron content is a basic figure for the calculation of all other pools and of all the flows of iron The present analysis gives a theoretical justification for this procedure Five different mechanisms which may be thought to be responsible for the variations of plasma iron content have been analysed by means of a simulation technique on a digital computer On the basis of each of these possible mechanisms theoretical radioiron curves were produced In a second step these curves were taken as representing actually observed experimental data Theoretical radioiron curves produced on the basis of a ferrokinetic model in *steady state* were then fitted to these "experimental data by adjusting the different parameters of the model The following results were obtained

1 Measurements of the so called "plasma iron turnover (actually the outflow of iron from the plasma at the time of the experiment) may be subject to considerable error if the disappearance rate of the tracer is recorded during a rather long period (3 hours) and plasma iron content is measured only at the beginning of the experiment The accuracy of the estimate is improved if the estimate of plasma iron concentration is based on the arithmetic mean value of serial determinations during the same period

2 Complete ferrokinetic studies including measurements of radioactivity in plasma and red cells during a period of 2 weeks are able to yield an accurate estimate of haemoglobin synthesis whatever the mechanism behind the diurnal variation of plasma iron content, provided that measurements of plasma radioactivity are performed only once a day and each day at the same time The radioactivity appearing in the red cells may be regarded as "averaged experimental data where daily fluctuations are eliminated by the processes of iron uptake in the bone marrow during a rather long period

3 Variations of plasma iron content from day to day may give

rise to a certain error in the estimate of the rate of haemoglobin synthesis if this estimate is based on an analysis of plasma radio activity (*cpm/ml* plasma on consecutive days) only This error can be eliminated by analysing the appearance curve and/or by using plasma radioactivity expressed as *cpm/μg* iron

4 Other ferrokinetic parameters especially the size of hypothetical small exchanging pools cannot at present be derived from radioiron data in plasma and red cells with a high degree of confidence

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ELIMINATION OF EXOGENOUS LIPIDS FROM THE BLOOD STREAM

AN EXPERIMENTAL, METHODOLOGICAL AND CLINICAL STUDY IN DOG AND MAN

BY

DAG HALLBERG

STOCKHOLM 1965

FROM KING GUSTAV V RESEARCH INSTITUE AND THE DEPARTMENT
OF SURGERY ST GÖRAN S HOSPITAL, STOCKHOLM SWEDEN

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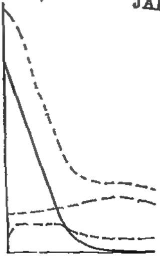

STOCKHOLM 1965

CONTENTS

This paper constitutes a summary of the following publications

I Studies on the elimination of exogenous lipids from the blood stream The kinetics of the elimination of a fat emulsion and of chylomicrons in the dog after single injection *Acta physiol scand 1963 59 52—61, in collaboration with Lars A Carlson*

II Studies on the elimination of exogenous lipids from the blood stream Determination and separation of the plasma triglycerides after single injection of a fat emulsion in man *Acta physiol scand 1964 62 407—421*

III Studies on the elimination of exogenous lipids from the blood stream The kinetics of the elimination of a fat emulsion studied by a *constant* infusion technique in man *Acta physiol scand 1965 63 (in press)*

IV Studies on the elimination of exogenous lipids from the blood stream The kinetics of the elimination of a fat emulsion studied by *single* injection technique in man *Acta physiol scand 1965 63 (in press)*

V Studies on the elimination of exogenous lipids from the blood stream The kinetics for the elimination of chylomicrons studied by single intra venous injections to man *Acta physiol scand 1965 (in press)*

VI Studies on the elimination of exogenous lipids from the blood stream The effect of fasting and trauma in man on the elimination rate of a fat emulsion injected intravenously *Acta physiol scand 1965 (in press)*

References to these papers will be made by the numerals I—VI
Abbreviation used TG = triglyceride(s)

INTRODUCTION

Lipids are substances in living material from one cell organisms up to man It constitutes, among other things a substance for storage and creation of energy In the microscope it is possible to observe the amoeba through phagocytosis ingesting a drop of oil and in every day life we see man taking meals rich in fats

Our knowledge about fat metabolism has increased considerably during the last decade The interest in lipid metabolism has been stimulated as it has become evident that there exist connections between lipid metabolism and several very common human diseases such as atherosclerosis and diabetes The formation of chylomicrons from fatty meals has been studied in mammals The chylomicrons of the chyle the physiological fat emulsion are formed in the intestinal mucosa enter the blood stream through the thoracic duct and then disappear into metabolic processes

Pathological states in man are sometimes accompanied by inability to ingest food by mouth To overcome this the technique for parenteral nutrition has been developed

This technique has improved the therapeutic results especially in that part of medicine where surgical procedures are indicated To assure a complete parenteral nutrition covering the caloric needs of the body we have to rely on lipids which are rich in calories This has resulted in the production of artificial fat emulsions some of which are now available for clinical use

The aim of the present investigation was

- To study and characterize the kinetics for the elimination from the blood stream of the TG of an artificial fat emulsion used for parenteral nutrition
- to compare the kinetic principle found for the TG of the artificial fat emulsion with that of the TG in the physiological fat emulsion the chyle
- to investigate in man the influence of fasting and surgical trauma on the elimination of the emulsion TG from the blood stream

Definitions

In this paper *exogenous* lipids refer to lipids in chylomicrons from the thoracic duct during absorption of dietary lipids or to lipids in artificial fat emulsion. If they reappear in plasma after having left the blood stream they are called *endogenous* lipids.

In the text of the papers (I—VI) constituting the basis for this dissertation no distinction was made between the expressions fat emulsion, fat particles of emulsion, and emulsion triglycerides (TG), e.g. artificial fat emulsion could signify the entire artificial fat emulsion, the fat particles of this artificial emulsion, or the TG of the emulsion. Chylomicrons refer either to chyle or to fat particles of the chyle, or to the TG of the chyle. These distinctions are sometimes essential but the correct meaning is usually evident from the context.

HISTORICAL BACKGROUND

Elimination of chylomicrons from the blood stream

The counting of chylomicrons in blood by means of the microscope was introduced by Gage in 1920 and Frazer and Stewart in 1937 Since then many contributions have been made to our knowledge of chylomicron metabolism

The first definition of chylomicrons was given by Gage (1920) He defines them as *microscopic bodies coming from the chyle* Dole and Hamlin (1962) specify them as *alimentary entities formed by intestinal cells during absorption of a fat meal* and Olivecrona (1962) uses the term chylomicrons for *particles carrying exogenous fat from the alimentary tract*

Different methods have been used for the determination of the amount of chylomicrons or their constituents The methods are based on different properties of the chylomicrons such as visibility in the dark field microscope light scattering in water solution density and chemical composition Isotopic tracer techniques have also been used Studies have also been performed with combinations of methods based on these properties

The term chylomicron" in blood has been used with various definitions and descriptions Albrink (1961) has thus described heavy" and "light" chylomicrons in *fasting* plasma with a centrifugation technique Kay and Entenman (1961) have found "chylomicron like" bodies produced by the *perfused liver* and Gordis (1962) by means of a density gradient has been able to describe two kinds of fat particles during *alimentary lipaemia* From these findings and the definitions given above it is obvious that the field has not been stabilized around strict definitions

The current concepts of the definitions and metabolism of chylomicrons have been reviewed by Dole and Hamlin (1962) and Olivecrona (1962)

After formation in the alimentary tract the chylomicrons are transported via the thoracic duct to the blood stream Then they disappear from the circulation The curve for the removal of chylomicrons from the blood stream has been described as following either a single exponential or a complex course (see I for references) A single exponential elimination rate a first order reaction means that the amount eliminated per unit of time is dependent on the concentration in the blood The rate of elimination of injected exogenous TG has also been reported to vary with the amount injected and to be inversely proportional to the dose (French and Morris 1957 Edgren 1960) But all attempts to give a simple explanation why an injected dose can determine the rate of a physiological phenomenon have so far failed

Mechanisms for the removal of chylomicrons from the blood stream have been reviewed e g by French Morris and Robinson (1958) and Dole and

Hamlin (1962) The two major mechanisms which have been discussed are pinocytosis of the chylomicrons and hydrolysis of the chylomicron TG by the enzyme lipoprotein lipase

Our present knowledge and the problems concerning the elimination of chylomicrons from the blood stream are well defined in the following quotation from Dole and Hamlin (1962) "The disappearance of particles from circulation has proven easier to measure than to interpret. In theory one should be able to estimate the rate at which native chylomicrons are removed by following the clearance of an intravenously injected test emulsion In practice, interpretation has been obscured by three difficulties First, the emulsion if made with abnormal surfactants or if it contains particles of abnormal size may be handled abnormally (Murray and Freeman 1951) different clearance rates are obtained with different kinds of artificial emulsions (Waddell et al 1953 a) Second the dose given in a single rapid injection may be greater than the amount leaving the thoracic duct during a comparable period of normal absorption the removal rate is dose dependent (Bierman and Hamlin 1962 Edgren 1960 French and Morris 1957) Third, particles removed by tissues may be recirculated in particulate form the material carrying label in plasma or contributing turbidity may be a mixture of different entities and thus obscure the primary clearance"

Artificial fat emulsions

Fat emulsions for clinical use were first produced in Japan in the 1920s (Yamakawa 1920 Nomura 1928) and since then, several kinds of emulsions have been produced in different countries The literature on artificial fat emulsions has been reviewed by Kauste (1958) Geyer (1960) Schön and Zeller (1962) and Edgren and Wretlind (1963)

The artificial emulsions had at first several toxic properties but by changing the composition and refining the components it has been possible to produce emulsions with few side reactions (Schuberth and Wretlind 1961 Edgren et al 1964) With the aid of fat emulsion it is now possible to keep dogs on complete parenteral nutrition during 10 weeks without obvious side reactions and maintenance of good health (Wretlind 1964)

The fat emulsions are composed of vegetable oils in water The emulsions are stabilized with a surfactant, e g phospholipids The size of the oil particles in some of the artificial emulsions is very close to that of the chylomicrons (Bierman and Hamlin 1962 Hallberg and Wersäll 1964)

Our knowledge on the metabolism of lipids in artificial emulsions has grown in parallel with our knowledge on chylomicron metabolism This is also true for the theories on the mechanism of their elimination from the

RESULTS

The kinetic principle found in the dog for the elimination of exogenous lipids (I)

A new artificial fat emulsion (Wretlind 1963) for parenteral nutrition with excellent clinical tolerance (Schuberth 1963) initiated this study on its elimination from the blood stream

Preliminary investigations in man with single injections of this emulsion with subsequent analyses of plasma total TG concentration gave results, which were difficult to characterize in a mathematical fashion (Hallberg unpublished data) A methodological study was therefore started in dogs The TG level in blood was followed after a single intravenous injection of the emulsion The preinjection TG content in the blood was subtracted from the total TG concentration in order to get an estimate of the amount of emulsion TG present in the blood In contrast to the findings in man the elimination course for the emulsion could be characterized in simple mathematical terms After the injection the TG concentration decreased linearly

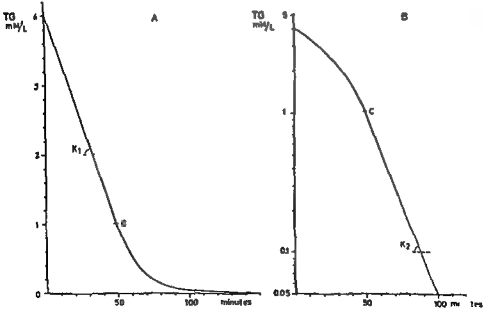

Fig 1

Constructed curve for the elimination from the blood stream of fat emulsion TG injected i v at zero time A in a linear graph B in a semilog graph

K_1 indicates the slope of the linear part of the curve in a linear graph (A)

K indicates the slope of the linear part of the curve in a semilog graph (B)

C indicates the concentration at which linear elimination (zero order reaction) changes into exponential elimination (first order reaction)

at high TG concentrations and exponentially at low concentrations (see fig 1) The elimination rate was independent of the dose A new kinetic principle for the removal of exogenous lipids from the blood stream had thus been observed

This observation prompted a comparison between the removal of the new artificial emulsion and the removal of chylomicrons isolated from chyle The TG in this physiological emulsion was found to be eliminated from the blood stream according to the same kinetic laws as the TG in the tested artificial emulsion

The interpretation of the results was that a maximal elimination capacity existed This capacity was designated by the constant K_1 and expressed as mmoles of TG per litre per minute The maximal capacity was utilized above a "critical concentration" (C mmole/l) Below this concentration the full capacity was not utilized and the amount of TG eliminated per minute was dependent on the TG concentration This elimination a fractional removal rate of exogenous TG was designated by the constant K_2 (per minute) The relation between these constants and the "critical concentration" was given a mathematical expression in the formula $K_1 = K$ C

Methodological studies on the separation of exogenous lipids from endogenous plasma lipids (II)

The elimination of emulsion TG in man was re-investigated by the same technique as that, described in the dog experiments The elimination curve was still too complex to be solved (Hallberg unpublished data)

If the process of elimination of exogenous lipids from the blood followed the same principle in the dog and man the complex curves seen in man could be explained as follows In man contrary to the dog, the basal (endogenous) level of TG may change considerably during the elimination of the emulsion TG If so, it was wrong to calculate the amount of emulsion TG present in the blood by subtracting the basal TG concentration from the total TG concentration in order to get an estimate of the amount of emulsion TG present in the blood To study whether there were any appreciable changes in the levels of endogenous TG during the elimination of exogenous TG in man it was necessary to find a method which separated exogenous TG from enogenous TG in human plasma

Model experiments with addition *in vitro* of the emulsion to plasma showed that it was possible with a centrifugation technique to choose conditions so that the emulsion TG was floated to the top of the centrifuge tube without affecting the *basal* plasma TG concentration With this separation technique the endogenous plasma-TG concentration found at the bottom of the centrifuge tube increased during the elimination of the

genous plasma-TG concentration so derived from the total TG concentration the elimination curves obtained for the emulsion TG were in some cases similar to the curves seen in the dogs but still complex in many cases This suggested that the TG in the top fraction after centrifugation, contained not only emulsion TG but also other TG probably of endogenous origin This problem was studied further

The density gradient method described by Gordis (1962) was applied to the problem of separating exogenous lipids from endogenous lipids *In vitro* experiments demonstrated that the gradient separated added emulsion TG quantitatively to a distinct layer at the top of the gradient tube The endogenous plasma TG remained in the lower part of the gradient and separated into two distinct fractions (middle and bottom fractions)

It was then found that the top fraction in the gradient was smaller than the centrifugal top fraction during the course of elimination of exogenous TG It was also demonstrated that the TG in the centrifugal top fraction was heterogenous when isolated and separated by the gradient method In the gradient they were found in both the middle and top fractions

The kinetic principle found in man for the elimination of exogenous lipids (II, III IV V)

The density gradient method was then applied to investigate the elimination of exogenous TG from the blood stream in man All curves obtained were consistent with the principle first found in the dog a linear rate above and an exponential rate below a "critical concentration" (see top fraction in figs 2 A and B)

Using a *constant* infusion technique (III) the results obtained by the *single* injection technique (II IV VI) were verified The elimination rate of TG found in the top fraction was independent of the given dose and did not vary during constant infusions of the emulsion for periods up to 3 hours (III—IV)

The mean and the standard deviation for obtained rate constants in subjects fasting overnight (III IV VI) was for the maximal elimination capacity (K_1) 0.07 mmole/l plasma/min ±0.03 mmole/l/min (S D n = 22) and for the fractional removal rate (K_2) 0.06 per min ±0.03 (S D n = 23)

Studies in 2 subjects injected with chyle isolated from the thoracic duct after fatty meals demonstrated that the kinetics for the elimination of chylomicron TG was the same as for TG from the artificial fat emulsion a linear elimination rate at high and a fractional removal rate at low concentration (V) The observed rate constants were of the same order of magnitude as for the artificial emulsion TG used

Effects of starvation and surgical trauma on the elimination of exogenous lipids in man (VI)

The purpose of giving artificial fat emulsion is to supply calories intravenously during periods of restricted caloric intake often in connection with surgical trauma It is therefore of importance to know whether the elimination of the emulsion is "normal during starvation and after trauma One group of patients known to be starving are those who cannot take food after abdominal surgery It is also essential to know whether these patients, known not to be metabolically normal eliminate the emulsion in the same way as do starving patients without surgery

The elimination was investigated in 2 groups of patients surgical and non surgical, after 15 hours and 39 hours of starvation It was found that the kinetic principle for the elimination was the same in both groups The nutritional state influenced the maximal elimination capacity (K_1) which had increased by 50 per cent after 39 hours of starvation The increase was 250 per cent after 39 hours of starvation in the surgery group The nutritional state did not change the fractional removal rate (K_2) but in the postoperative state K_2 increased by about 80 per cent

Recirculating lipids (III—VI)

During the elimination of both the artificial and the physiological emulsions from the blood stream in man, considerable changes were observed in the endogenous TG found in the middle and bottom fractions in the density gradient (fig 2) Some information was obtained concerning the kinetical relationship between these changes and the elimination of the emulsion TG from the blood stream The magnitude (b in fig 2 B) and duration (a in fig 2 B) of the changes in the middle fraction TG seem to depend on the given dose of fat emulsion (III—IV) but also on the nutritional and traumatic state of the recipient (VI) The results suggested that the emulsion TG is not an immediate precursor responsible for the change in middle fraction TG (III IV) During constant infusion of high doses of the fat emulsion the concentration of middle fraction TG increased continuously which indicates that more middle fraction TG was formed than what was eliminated At low doses however an equilibrium was obtained (fig 2 in III) The kinetic principles for the middle fraction TG seem to be of the same nature as those for the emulsion whereas the kinetics for the bottom fraction TG is not clear from this study

From a kinetical view point it was supposed in this study that the product from a reaction at a constant rate (zero order reaction) in one pool (the pool of the precursor) could act as a constant infusion into another pool (the pool of the product) (IV) It was presumed that this event was represented by the plateau level of the middle fraction TG concentration an observation in many cases during the zero order elimination for the top fraction TG (see fig 2)

The density gradient method is still too new to permit an evaluation of the physiological significanses of the three different fractions The chylomicrons in the top fraction and the lipoproteins in the bottom fraction are rather well known entities It is unknown whether the middle fraction TG (the secondary particles of Gordis 1962) are aggregates of TG rich lipoproteins or a particulate physiological reality Whether this fraction consists of aggregrates of lipoproteins or not, it acts like a kinetical unit separated from both the top and the bottom fraction TG

A possible mechanism for the elimination of exogenous lipids from the blood stream

The kinetic principles for the elimination of exogenous lipids observed in this study was expressed in mathematical terms The formula given has not been described earlier in the available literature on the process of elimination The formula $K_1 = K_2 \cdot C$ (see page 10 for explanation) consists of the symbols for two rate constants and one concentration

With this formula as a basis it is tempting to try to explain the mechanism for the elimination of exogenous lipids from the blood stream *In vivo* many reactions are mediated by enzymes and it is possible that an enzyme reaction may be a controlling step in the removal of exogenous lipids The following arguments will support this hypothesis

From an enzymatic viewpoint a zero-order reaction (K_1 *mmole/l/min*) means that all enzyme is occupied (saturated) by the substrate (Dixon and Webb 1960) In this case the substrate should be the exogenous lipids The "critical concentration" (C mmole/l) in this study then *indicates* the optimal substrate concentration necessary to reach the maximal reaction capacity with available enzyme(s) K_1 may thus be a measure of the amount of available enzyme

As mentioned above lipoprotein lipase may be the enzyme system associated with the initial removal of exogenous lipids In connection with the hypothesis it is interesting to note a study on human heparin induced lipoprotein lipase enzyme by Boberg and Carlson (1964) The fat emulsion used by them as a substrate was the same as that used in this study They had found a maximal enzyme activity in plasma after heparin

which hydrolyses the emuls on at a rate of 0 20 μmoles of fatty acid per ml plasma and minute This corresponds to complete hydrolysis of 0 07 mmole of TG/l plasma and minute which is the figure found in this study for K_1

If the site(s) of elimination is *not* evenly distributed in the body then the blood flow passing the site(s) is one factor of importance for the rate of elimination *below* the "critical concentration" This rate will appear as a fractional removal rate (K_{-} per minute) the magnitude of which is related to that fraction of the total blood volume (or total volume of distribution of the substrate) which per unit of time passes the eliminating site(s)

Above the "critical concentration" the capacity of the enzyme is fully utilized and the size of the blood fraction passing the enzyme per unit of time will be of no importance to the elimination rate

At the "critical concentration" the amount of TG in this blood fraction will be equal to the capacity of the enzyme to combine with the substrate

The clinical study

In clinical work there are many problems unsolved concerning the physiology and the pathology of exogenous and endogenous lipids The fat emulsion was used in a test load to get an answer whether the nutritional state does influence the removal rate of the exogenous lipids from the blood stream Another question of great clinical interest is how the operated patients the candidates for parenteral nutrition do eliminate the fat emulsion TG from the blood stream

Exogenous lipids The clinical application (VI) of the kinetic principles found in this study showed that the maximal elimination capacity of exogenous lipids increased during starvation and more so after surgical trauma

The average value for K_1 after fasting overnight (15 hours) was 0 07 mmole/l plasma/min ±0 03 mmole/l/min (S D) Let us assume the molecular weight of the TG to be 885 and the plasma volume to be 43 ml/kg body weight The maximal elimination of exogenous TG from the blood stream during a 24-hour period can thus be calculated as follows if we also assume that K_1 remains constant during the 24 hour period

0 07 885 10^{3} 0 043 60 24 = 3 8 g (±1 6 g S D) TG/kg bodyweight/24 hours The figure corresponds to 35 calories/kg/24 hours From a practical viewpoint it is noteworthy to observe the standard deviation for the calculated mean value and also that the mean value is valid only after 15 hours of fasting The corresponding value after 39 hours of fasting is 52 calories/kg/24 hours and after a surgical trauma with a 39 hour fasting period the value is >100 calories/kg/24 hours An expenditure of 8 000

9 000 calories/24 hours has been observed in man during heavy work (cf Rein 1948) The figure corresponds to about 125 calories/kg/24 hours in a person with a body weight of 70 kg

Therefore it is perhaps more than a guess to suppose that the high maximal elimination capacity of exogenous lipids observed after surgical trauma is an answer to a high caloric need

The fractional removal rate (k_2) of exogenous TG did not change during fasting but it increased after surgical trauma It has been reported that the nutritional state does not influence the removal rate of chylomicrons from the blood stream (for references see VI) It is therefore tempting to suppose that earlier investigations were performed below the "critical concentration" and without changes in the traumatic state

The observations on the changes of the magnitude of the two rate constants will be interpreted from the viewpoint of the hypothesis for the elimination mechanism The mechanism (amount of available enzyme) may change with the nutritional state and the blood perfusion through the eliminating organ(s) may increase in the traumatic state

A zero-order reaction in an organism means that it works at the upper limit of its capacity This limit may be determined by the amount of available enzyme(s) The changes observed for k_1 during different nutritional state may be explained by changes in the amount of available enzyme It has been shown that lipoprotein lipase activity in plasma (Sandhofer et al 1962) and in different organs (Hollenberg 1959 1960 Robinson 1960 and Salaman and Robinson 1961) changes with the nutritional state Moreover it is shown that the nutritional state is of importance for the rate of the hydrolysis of chylomicron TG in adipose tissue and that the rate has an upper limit (zero order reaction) (Rodbell and Scow 1965)

Changes have been observed in the fractional removal rate of indocyanine green from the blood stream during mild and maximal exercise in man (Rowell Blackmon and Bruce 1964) The fractional removal rate of the dye decreased during exercise which is known to reduce the hepatic blood flow The liver is known to be the eliminating organ of indocyanine green

The lower fractional removal rate observed for emulsion TG in splenectomized dogs (Waddell et al 1953 b and paper I) may be due to a diminished portal blood flow

The increase of the fractional removal rate observed in the operated subjects was accompanied by an increase of the pulse rate and by an unchanged blood pressure This increase of the pulse rate argues for an increased cardiac output and an increased blood perfusion through the eliminating organ(s)

Endogenous lipids It was observed that the endogenous TG concentration in plasma was subjected to changes during the test load with exogenous

lipids in man The size and duration of these changes were dependent on the dose of exogenous lipids injected, and also of the nutritional and traumatic states of the subjects

It has been shown earlier that starvation and surgical trauma in man are accompanied by an increased utilization of the fat depots (cf Moore 1962) How this utilization is accomplished is not fully clarified, but it involve changes in free fatty acids and endogenous plasma-TG concentrations (Wadström 1959)

The nutritional state is reported to influence the endogenous plasma TG in a complex manner (for reference see VI) Wadstrom (1959) has shown that surgical trauma in man is accompanied by a decreased (endogenous) TG level in plasma.

If the hypothesis for the elimination of exogenous TG is valid also for the endogenous TG it is possible to explain Wadstrom s finding of a decreased glyceride level by an increased circulation through the eliminating organ(s)

The magnitude and duration of the changes in endogenous plasma TG observed in this study are compatible with either an increased turn-over rate or a decreased formation of endogenous TG after prolonged fasting and surgical trauma. In view of the enhanced elimination of exogenous TG in these states the linked kinetical relationship observed between exogenous TG, and the reported utilization of the fat depots an increased turn-over rate during the prolonged fasting and surgical state is the more plausible alternative

SUMMARY

1 A brief review is given of the concepts and problems in the earlier literature on the elimination from the blood stream of chylomicrons and artificial fat emulsions both of which represent exogenous lipids

2 The kinetics for the elimination from the blood stream of triglycerides of an artificial fat emulsion and chylomicrons was studied in dogs and human subjects

3 A new kinetic principle for the elimination was observed It was characterized by a zero-order reaction at high and a first-order reaction at low concentrations of triglycerides in plasma The zero-order part of the elimination was interpreted as a maximal elimination capacity, designated as k_1 (mmole/l plasma/min) operating above a "critical concentration" C (mmole/l plasma) Below this concentration there was a fractional removal rate designated as k_2 (per min) The relationship between these constants was given a mathematical expression in the formula $k_1 = k_2 \ C$

4 No differences were observed in the kinetic principle between the elimination of triglycerides from chylomicrons and those from an artificifical fat emulsion Nor were there any qualitative differences in this respect between dog and man

5 It was necessary to separate the emulsion triglycerides from endogenous plasma triglycerides in order to establish the kinetic principle in man Methodological studies showed that it was impossible to perform this separation by centrifugation techniques However a density gradient technique described by Gordis was found to make this separation possible

6 The kinetic principle was verified in man both by constant infusions and by single injections of exogenous lipids

7 The following quantitative results for the rate of elimination were obtained

   a The rate of elimination was independent of the dose

   b The maximal elimination capacity was influenced by the nutritional state of the subjects and increased during starvation

   c After abdominal surgery the maximal elimination capacity increased still more than after fasting The fractional removal rate also increased significantly

8 During the elimination of the exogenous triglycerides from the blood stream considerable changes were observed in endogenous plasma triglycerides

9 The formula that describes the elimination from the blood stream was used as a basis for a hypothesis on the mechanism of removal The essential in the hypothesis is an enzymatic reaction localized some-

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STUDIES
THE EFFERENT VAGAL CONTROL OF THE STOMACH

BY
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GÖTEBOPG 1965

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THE EFFERENT VAGAL CONTROL OF THE STOMACH

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The present summary is based on the following publications

I Excitatory and inhibitory effects of vagus stimulation on gastric motility in the cat Martinson J and A Muren Acta physiol scand 1963 *57* 309—316

II The effect of graded stimulation of efferent vagal nerve fibres on gastric motility Martinson, J Acta physiol scand 1964 *62* 256—262

III Some quantitative considerations on vagally induced relaxation of the gastric smooth muscle in the cat Jansson, G and J Martinson Acta physiol scand 1965 *63* 351—357

IV Vagal relaxation of the stomach Experimental re-investigation of the concept of the transmission mechanism Martinson, J Acta physiol scand 1965 In press

V The effect of graded vagal stimulation on gastric motility secretion and blood flow in the cat Martinson J Acta physiol scand 1965 In press

These papers will be referred to in the text by their Roman numerals

The English was checked by L. James Brown

Introduction

The stomach has a complex function. It acts as a receptacle for food and as an organ of digestion by converting the food into chyme, which is emptied successively into the duodenum. These functions are under the influence of delicate neural and hormonal control mechanisms. Though advances have recently been made in our knowledge of the regulation of gastric acid secretion (see Gregory 1962 p. 40 ff and Olbe 1964), the complex regulating mechanisms of the stomach are in many other respects still obscure. The central control is exerted by the two divisions of the autonomic nervous system. The parasympathetic outflow arrives at the stomach mainly *via* the vagus nerves, though some observations suggest that parasympathetic fibres also reach the stomach *via* the splanchnic nerves (for ref. see Semba, Noda and Fujii 1963). The sympathetic outflow to the stomach is derived from the coeliac ganglion and from the splanchnic nerves.

The major influences of the extrinsic nerves on gastrointestinal motility have been known for more than a century. The pioneers in this field are referred to in the classical study by Bayliss and Starling (1899), who expressed the opinion, prevailing ever since, that the vagus and splanchnic nerves exert a reciprocal control of the motility of the gastrointestinal tract. In this control system the vagus exerts the excitatory influence but there are many observations indicating that the vagus also inhibits motility. The latter has been consistently observed on the stomach (see McSwiney 1931). Otherwise the control of gastric motility is generally considered to be similar to that of intestinal motility.

That gastric secretion is controlled by the vagus was shown by the Pavlovian school (Pavlov and Shumov-Simanovski 1890). The role of the splanchnic — sympathetic — nerves is less clear with regard to the gastric secretion. The sympathetic influence has been supposed to be inhibitory but evidence produced has never been convincing (for ref. see Emås 1964).

The vagal parasympathetic fibres are generally regarded as presynaptic, making ganglionic contacts with the intrinsic nerve plexa. Recent observations (Norberg 1964) indicate synaptic connection also between the sympathetic fibres and the nerve cells of the submucous and myenteric plexa, a view arrived at also by Hewenter (1965) with respect to the intestine, an arrangement which might be of considerable functional significance.

It is not properly understood how the local nerve cells are interconnected or how they are connected to the extrinsic nerves In view of the smallness of the total number of fibres in the vagus nerves compared with the presumably much larger number of ganglion cells in the visceral wall Langley (1922) assumed a considerable degree of divergence It is still not known whether each intrinsic nerve cell is innervated by preganglionic vagal fibres, but the discrepancy between the number of vagal fibres and the number of nerve cells in the walls of the viscera is in fact larger than that suggested by Langley for we now know that the number of efferent fibres in the abdominal vagus of the rabbit and of the cat is only about 3 000 or less than 10 % of the total count (Evans and Murray 1954 Agostoni *et al* 1957)

Efferent fibres in the vagus nerves are derived from the dorsal motor nucleus of the vagus (Ranson and Clark 1959 p 248) The efferent vagal neurones are under the influence of cortical and subcortical areas in the central nervous system (for ref see Eliasson 1960)

Afferent nerves from the stomach reach the central nervous system *via* the vagus and the splanchnic nerves There is evidence suggesting that the vagal afferent fibres carry information from mechanoreceptors and chemoreceptors (Paintal 1954 Iggo 1957) in the stomach wall whereas the splanchnic nerves carry information to the somatosensory cortex, presumably from nociceptors in the viscera (*cf* Downman and Evans 1957)

The effect of stimulation of the vagus nerves on gastric motility has been the subject of fairly extensive studies (for ref see McSwiney 1931) That vagal stimulation does not always enhance, but also sometimes inhibits motility had at that time been known for several years, but ... ssion of the cause of this inconsistency became more extensive ... by McCrea, McSwiney and Wadge (1925) and by V ... who arrived at conflicting conclusions about ... Veach stressed the importance of the ... finally (Veach, Schwartz and Weinstein 1 ... tion was simply "inhibition through fatig... around McSwiney (McCrea, McSwiney an... Wadge 1928 Brown and Garry 1932) ... gastric response to vagal stimulation w... excitation occurred when the tone was ... form a clear concept of the "peripheral ... ne... i e a mechanism causing excitation ... another

Aim of present studies

It was considered of interest to re evaluate the effect of nerve stimulation on gastric motility In studies I and II attention was focused on the effect of variation of the vagal stimulation parameters on the motility of the stomach

The results of these studies made it desirable to study some of the responses in further detail Study I shows that the vagus nerves contain not only fibres enhancing gastric motility but also fibres which relax the stomach Recent investigations (Greff, Kasperat and Osswald 1962, Paton and Vane 1963) have suggested that vagal inhibition is exerted by some adrenergic mechanism Preliminary results obtained in experiments of the type described in I where the animals had been pretreated with reserpine were however not compatible with the idea of an adrenergic transmission The transmission mechanism of vagal inhibition was therefore studied with special reference to the suggested adrenergic mechanism (IV)

Different methods of studying gastric motility have been used by different workers Most methods give only crude information in terms of excitation or inhibition tone or pressure waves In a search for the functional meaning of the different motor effects observed in study I and II, among other methods direct visual inspection and measurement of the gastric volume were tried The problems of the real gastrointestinal motor functions can certainly not be solved in the anaesthetized animal, and especially not by means of efferent nervous stimulation Nevertheless the recording of gastric volume and also to some extent visual inspection of the stomach movements were considered to give information about one of the gastric functions *viz* its function as a receptacle for food The results also shed new light on the old observation of the role played by the initial tone as a determinant of the gastric response to vagal stimulation The results are presented in III

It was hoped that the grading of stimuli in I would give information also about the fibres in vagus nerves which control gastric secretion Do the excitatory or inhibitory vagal fibres for the control of motility also control secretion in a similar way? Do the secretory nerve fibres form a separate group? An attempt to answer these questions is presented in V

Gastric blood flow has recently received attention (for ref see Jacobson 1963) Interest seems to have been directed mainly to the role of the gastric blood flow as a factor limiting secretion Conversely the possibility that an increase in gastric blood flow should *per se* stimulate gastric secretion has also been discussed (Jacobson Scott and Frohlich 1962) But since the

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Results and discussion

1 Efferent fibre groups in the vagus nerves

Results reported in I and V indicate that the vagus nerves contain two groups of efferent nerve fibres controlling different gastric functions. These two groups could be separated by their properties of excitability. Almost all efferent nerve fibres in the vagus nerves are thin and non-myelinated (Evans and Murray 1954, Agostoni *et al* 1957). Such fibres presumably respond only to strong stimuli and their conduction velocity is relatively low. There are no available data suggesting a morphological division of the efferent fibres in various subgroups.

When applying stimuli of graded strength or impulse duration the activation threshold found for each group of fibres suggested a strength-duration correlation of the kind described by Lapicque (1929). This first division into two fibre groups, one exciting, the other inhibiting gastric motility (I) could subsequently not be extended to hold also for other gastric function, *i e* that they too were controlled by groups of fibres distinctly different from those controlling motility. Instead, the high threshold fibre group clearly inhibiting motility was shown to include fibres with identical threshold properties stimulating the secretions of hydrochloric acid and pepsinogen in the stomach and causing vasodilatation (V).

It has been adequately shown (Kay and Smith 1951) that the fibres eliciting stomach contractions and secretion are preganglionic. The inhibition of gastric motility is also mediated by preganglionic vagal fibres (IV). The various gastric responses elicited on stimulation of the efferent vagal fibres can differ characteristically owing to differences in the *post-ganglionic* neurones. It should therefore be pointed out that the electrophysiological properties observed hold only for the preganglionic efferent vagal fibres. Pharmacological and other differences of the postganglionic neurones and their subordinate effector cells can hardly be used for further differentiation of the nervous pathways by preganglionic stimulation unless there is a distinct one-to-one ratio between the preganglionic and postganglionic neurones. The question arises whether one and the same fibre might, in fact, always elicit a peripheral pattern of responses comprising secretion, vasodilatation and relaxation of the muscle wall. Some evidence

for such a type of arrangement is discussed in the final section of this chapter

In the autonomic nervous system the control of the effector cells is maintained by impulses discharged at a relatively low rate in the nerve fibres (Rosenblueth 1952, Folkow 1955, Uvnäs 1960 and Hillarp 1960). In II it was shown that both the low-threshold excitatory and the high-threshold relaxing fibres can exert virtually their full range of control of gastric motility at impulse frequencies of less that 10 imp/sec. In V a similar observation was made concerning the secretion and vasodilatation, i.e. the impulse frequency of nerve stimuli producing maximal responses was to be found in the vicinity of 10 imp/sec.

2. Vagal control of gastric motility

The characteristics of the gastric motor responses to graded vagal stimulation are described in I, II and III. The low-threshold group of fibres act upon the gastric smooth muscles with the classical vagal effect — enhancement of gastric motility. The larger the number of fibres excited (I, Fig. 2 and 3) or the higher the impulse rate up to the level of some 10 imp/sec (II, Fig. 2) the stronger the responses.

The vagal excitatory responses were most readily recorded with the balloon technique. As the experiments in I and II were performed without abdominal surgery — the balloon was passed down *via* the oesophagus — it might be assumed that this was responsible for the enhancement of the excitatory responses. A laparotomy is by itself considered to depress gastro-intestinal motility (*cf.* Hughson 1925). In subsequent experiments (V) it was found that the gastric excitatory motor responses to vagal stimulation were smaller after the abdomen had been opened though qualitatively the same. With the volume recording technique used in III excitatory responses were not so readily obtained when the intraluminal pressure was low or when the stomach volume was small. At higher intraluminal pressures and in a relaxed state, *i.e.* after stimulation of the high threshold fibres, the stomach responded more readily with a decrease in volume — enhanced motility — to low-threshold vagal stimulation (see *e.g.* III, Fig. 2). However, the balloon was always filled with about 30 ml of air, a volume which in view of the results with the volume-recording system must have distended the stomach and thereby facilitated excitation of the gastric smooth muscles.

The gastric motor responses to stimulation of the high threshold group of fibres in the vagus further illustrate the difference between different

recording techniques The principal differences are to be found in the pressure volume relationships While the balloon displaced 0 3 ml/cm H_2O, the volume recording system was principally designed to displace infinitely large volumes at very small pressure changes In most reports on gastro-intestinal motility the recording system used was of the type in which large changes in pressure resulted in displacement of only small volumes It is in this respect probably less important whether a ballon system or a fluid -filled system is used provided the balloon is large enough to fill the stomach Although the inhibitory responses were clearly visualized when recorded with a balloon the magnitude of the response proved many times larger when recorded in terms of gastric volume (III) In fact these fibres were able to produce a manifold increase of the gastric volume provided that the relaxation started from a state of high tone in the stomach On the other hand when the tonus of the stomach had been lowered by vagal stimulation subsequent stimuli produced no further relaxation (*cf* III Fig 2) Then only excitatory responses could be elicited These findings explain why the vagal stimulation is excitatory when the tone is initially low, and inhibitory when tone is high (McCrea McSwiney and Stopford 1925) Tone is to be understood here as the state of contraction of the stomach muscle It is however necessary to distinguish between pure myogenic tone and the tone set up by continuous activity of the nervous elements controlling the effector It was observed in IV that neither atropine nor hexamethonium had any significant effect on the basal tone This indicates that the vagally decentralized stomach subjected to low intraluminal pressure is not influenced by continuous nervous discharge from excitatory or relaxing nerve elements

Harper Kidd and Scratcherd (1959) reported an increasing basal tone of the stomach after vagotomy in the acute experiment an observation also often made in the present experimental series This observation indicates a tonic central influence *via* the specific relaxing nerve fibres when the vagus nerves are intact

When the vagi are intact an important tonic influence is presumably exerted also *via* the excitatory fibres Inhibition of gastric motility could then be brought about by inhibition of this nervous tone *i e* with a mechanism differing from that of the high threshold vagal fibres which apparently act by specificly relaxing the stomach smooth muscles Inhibition of excitatory nervous tone might also constitute an important principle in the control of gastric motility Inhibition of this kind can furthermore be accomplished in two ways the excitatory preganglionic neurone can be inhibited within the central nervous system or the postganglionic

neurones can be inhibited within the stomach wall. This latter type of inhibition mechanism is suggested by Kewenter (1965) to be accomplished by the sympathetic fibres to the gut and it has its anatomical correlate in the sympathetic terminals surrounding the cells of the submucous and myenteric plexus observed by Norberg (1964). Preliminary observations have made a similar organisation within the stomach highly probable (Jansson and Martinson to be publ.)

A possibility considerably to increase the volume of the stomach by means of specific relaxing nerve fibres seems highly meaningful. The vagal relaxing fibres may thus be supposed to fulfill an important function when the stomach receives a meal, *i. e.* in the receptive relaxation. The corpus-fundus part of the stomach serves as a receptacle for ingested food. In III it was observed that the vagal relaxing fibres act predominantly upon the corpus-fundus. Little or no relaxing effect could be observed in the antrum. The motility of the antrum seemed to be enhanced by all types of stimuli. The receptive relaxation has been described as a phasic event, an inhibition of motility in the corpus-fundus correlated in time to the act of swallowing (Cannon and Lieb 1911, Lind *et al.* 1961) or as the ability of the stomach to change its size within wide limits without much increase in intragastric pressure (Grey 1918) and has been shown to be dependent on the vagal innervation of the stomach. But also local reflex systems may be involved, for increasing intragastric pressure causes a release of gastric muscle tone when pressure reaches about 4 cm of water, an observation largely corroborated by the present experiments (*cf.* Paton and Vane 1963 and III, Fig. 4 A). It is however not yet possible to decide whether this "fundal release", as termed by Paton and Vane, is a manifestation of the postganglionic part of the vagal relaxing system.

The transmission mechanism of vagally induced relaxation of the stomach. It was suggested that the gastric relaxation brought about by vagal stimulation might be a specific response elicited *via* some mechanism acting upon the smooth muscle cells with consequent marked relaxation of the corpus and fundus. Since this relaxation was not abolished by atropine, it was earlier regarded as adrenergic. In study IV the vagal relaxation was, however, found to differ in several respects from the responses induced by sympathetic stimulation or catecholamines. The vagal response is generally stronger, it develops more rapidly and wears off *less* rapidly than adrenergic responses. Furthermore, it cannot be blocked by anti-adrenergic drugs such as guanethidine or α- and β-adrenergic blocking compounds. These findings are incompatible with those made by Greeff, Kasperat and Osswald (1962).

and by Paton and Vane (1963) on isolated stomach preparations. Their results favoured the conclusion that vagal inhibition *is* due to adrenergic fibres in the vagus nerves. Various explanations for this discrepancy are offered in IV. It is therefore concluded that the vagal inhibitory or, preferably *relaxing* nerve fibres are not adrenergic. The adrenergic fibres in the vagus and the splanchnic nerves might be concerned with some other function regulating gastric smooth muscle and seem, as mentioned mainly to exert their action on the intramural nerve cell plexus with supression of excitatory nervous tone. This is still an important difference for the vagal relaxing nerve fibres can be said if anything to potentiate the excitatory influence, which increases with the degree of relaxation of the stomach.

The transmitter substance finally responsible for the long lasting potent relaxation elicited *via* vagal fibres is still unknown. Neither is it known with certainty whether there is any single transmitter in the conventional meaning of the word. It would appear that the characteristic prolonged relaxation, a new tonus state of the stomach muscles must be ascribed to some unknown complex system outside or within the muscle cells. It seems less likely that the intrinsic nerve plexus forms any system which for the very long time observed *e g* by reverberating activity, would keep the stomach smooth muscle in a relaxed state.

It was considered worth while to test some biogenic substances such as histamine 5 hydroxytryptamine bradykinin and gastrin which often though not invariably have some relaxing effect on the stomach. None of the substances can mimic the powerful vagally elicited gastric relaxation. Antihistaminics and antiserotonins have no blocking effect. Extracts of the stomach have not yet been tried but cross circulation experiments have failed to demonstrate any circulating substance with the properties sought being released from the stomach.

The very slow elimination of the vagally induced gastric relaxation contrasts to almost all other types of nervous effector responses. Only the vasodilatation elicited by antidromic activation of thin afferent nerve fibres in somatic nerves show some similarity in this respect. This type of vascular smooth muscle relaxation also wears off extremely slowly (*cf* Celander and Folkow 1953) and here too the effect is elicited *via* some unknown transmission mechanism (Holton and Perry 1951). However the vagal relaxation of the stomach cannot be explained by antidromic activation of afferent fibres because the vagal relaxing mechanism includes ganglionic transmission steps the vasodilatation elicited *via* fibres with the same excitability is *not* prolonged and finally the frequency response correlation for the vagal relaxation is quite different from that for the anti-

dromic vasodilatation The latter can be brought to maximal levels by only a few subsequent stimulation pulses even at frequencies below 1 imp/sec.

3 Vagal control of gastric secretion

This section is concerned with the findings reported in V It was found that the high threshold group of nerve fibres eliciting gastric relaxation also excited the secretory functions of the stomach In all other respects the findings reported in V are in agreement with what has long been known about gastric juice produced by vagal stimulation Such gastric juice is rich in both acid and pepsin (see Babkin 1950 e g p 219 ff)

In V interest was focused on the presumed direct vagal control of the secretion of hydrochloric acid and pepsin The vagal control of mucus secretion was not studied Further the study did not take into account the important interplay between the neural and the hormonal mechanisms controlling gastric secretion of hydrochloric acid (for ref see Gregory 1962 p 40 Olbe 1964) This means that it was not possible in V to ascertain whether also the antral gastrin-producing cells are innervated by the high-threshold vagal fibres

In V a latency of 5—10 minutes was noted between the moment stimulation was started and demonstration of secretory response This interval has also been reported in other studies and its cause has been discussed (Babkin 1950 p 220 and Gregory 1962 p 17) However one report (Klopper 1954) seems to have passed more or less unnoticed When directly recording intragastric pH presumably the pH of the mucosal surface Klopper found a secretory response (decrease of pH) within 15—30 sec of the onset of stimulation of the brain cortex within the sigmoid gyrus The same latency was reported by Eliasson (1952) for the motility responses (excitatory and inhibitory) elicited from the same area. In view of Klopper's observation it would seem that the latency found for acid secretion was in reality due to some lag of the recording technique The short latency of the vasodilatory response to vagal stimulation (V and next section of this chapter) is also a sign of an ordinary latency of the gastric secretory neuroeffector system provided that vasodilatation is secondary to secretory work or as one would expect, at least correlated with such work

4 Vagal influence on gastric blood flow

In V it was observed that stimulation of the high threshold vagal fibres also increases gastric blood flow This vasodilatation is looked upon as a

functional vasodilatation i e a vascular response to meet the increased metabolic requirements of an active effector organ in this particular case the secretory work of the gastric mucosa It may be regarded as established that the metabolism of the activated gastric muscular wall does not call for any significantly increased blood flow (Kewenter 1965 and V)

The resting gastric blood flow was on the average 18 ml/min × 100 g tissue at a mean arterial pressure averaging 100 mm Hg During maximal vagal activation the blood flow increased to about 45 ml/min × 100 g During maximal vasodilatation induced by large doses of isopropylnoradrenaline the vascular bed of the stomach allowed a blood flow of 100—130 ml/min × 100 g Although these values on blood flow are 2—3 times larger than for skeletal muscle they are considerably lower than for intestinal blood flow (Folkow Lundgren and Wallentin 1963) This difference might be explained at least in part by the different functions of the organs presumably in the resorptive function of the gut

With the experimental technique used in V it was only possible to measure the blood flow in the entire stomach Since this is composed of roughly equal amounts of mucosa and muscle and since presumably only a small fraction (10—20 % of resting flow) of blood flows through the muscular part both in resting and active conditions it must be assumed that the flow through the mucosa is considerably higher than that recorded Furthermore the functional vasodilatation occurs only in the actively secreting mucosa (Menguy 1962) Taking all this into consideration the calculated blood flow in the corpus fundus mucosa during maximal secretion can be approximately deduced to reach figures may be as high as about 150 ml/min × 100 g

The total gastric blood flow values given in the litterature vary widely (for ref see Grim 1963) and recently published values have widened the range still more Thus Cumming *et al* (1963) reported a resting blood flow, measured by the venous outflow technique in the anaesthetized dog, of 14 ml/min × 100 g Delaney and Grim (1964) reported flow values of 53 ml/min × 100 g in the awake dog measured with a K^{42} clearance technique controlled by venous outflow measurements But they gave no information about the secretory activity in the stomach These observations suggest important differences in experimental design both as regards anaesthesia and surgery on one hand and the method of recording blood flow on the other

That the gastric mucosa becomes hyperaemic when secretory active is a very old observation in gastrointestinal physiology (Beaumont 1833 p 106) Burton Opitz (1910) Lim Necheles and Ni (1927) and Boenheim

(1930) were unable to show any effect of vagal stimulation on the total gastric blood flow However, none of these authors recorded gastric secretion and their observed blood flow values were remarkably high at least when compared with the present findings suggesting that the vessels of the stomach were already dilated Wolf and Wolff's (1943) experiments showed that the human gastric mucosal blood flow increased in association with phsychic stimulation of gastric secretion

Considerable interest has been devoted to the vascular architecture of the stomach (Barlow 1952 Boyd 1952, Walder 1952) and the existence and physiological significance of arteriovenous anastomoses have been discussed Evidence of anastomoses which can divert blood from the gastric mucosa and redistribute it is available (Peters and Womack 1958) A redistribution of blood flow to active parts of the gastric mucosa occurs during secretion (Menguy 1962) However whether this is a matter of shunting through arteriovenous anastomoses, is far from clear or according to our present concept of the control of blood vessels not even probable

The vagal vasodilatation in the stomach may be due to specific vasodilator fibres Also the latency of vagal vasodilatation is so short that it is compatible with the assumption of a direct nervous mechanism Such vasodilator nerves need not be of central origin they may be nerve cells situated within the intrinsic plexus and centrally controlled by fibres of the high threshold group of vagal fibres There is no evidence of cholinergic vasodilator nerves supplying the intestine neither in the splanchnic nerves (Bulbring and Burn 1936 Gernandt and Zotterman 1946) nor in the vagus nerves (Celander and Folkow 1953)

One might also imagine the vasodilatation to be secondary to gastric secretion In many respects this problem resembles that of functional vasodilatation in the salivary glands Barcroft (1914) suggested the vasodilatation in glands to be directly correlated with their rate of metabolism Terroux Sekely and Burgen (1959) refutes Barcroft's assumption of a direct correlation between oxygen consumption and blood flow However also a specific mechanism involving formation of so called plasma kinins has been proposed to account for the functional vasodilatation in glandular tissue (Hilton and Lewis 1956)

The vasodilatation seen in the stomach on vagal stimulation is not of the same order of magnitude as the functional vasodilatation in salivary glands It was frequently observed however that after a dose of atropine (0 1—0 6 mg/kg) high threshold vagal stimulation still produced some dilatation (15—30 %) i e the ability of the vessels to dilate was reduced but not abolished by atropine in a dose large enough to abolish neural

gastric secretion Since this is qualitatively similar to the situation in the salivary glands it was thought that a plasma kinin mechanism might be at work also in the stomach Tyrode s solution, perfused through the vascular bed of the stomach, and gastric juice were therefore tested for their content of plasma kinin forming enzymes Such enzymes were found occasionally (Jansson and Martinson 1963), but in such small quantities compared with those obtained from salivary glands by Hilton and Lewis (10^3—10^5 times less) that they were considered insignificant It was concluded that the method of Hilton and Lewis for detecting plasmakinin forming enzymes is not suitable for detecting the existence of such enzymes if any, in the stomach

5 Considerations on vagal control of gastric response pattern

The experiments presented have shown that the vagus nerves contain 2 groups of efferent fibres that can be separated by their electrophysiological properties These fibres are preganglionic and thus control a complicated intrinsic nerve plexus within the stomach wall The fibres possibly originate within the dorsal nucleus of the vagus (*cf* Laughton 1929) but recent observations by Semba *et al* (1964) suggest a different site of origin for the fibres inhibiting gastric motility The latter observation might be of importance for the further analysis of the response pattern discussed here

Part V presents a hypothesis according to which the high-threshold fibres form a uniform functional group where each fibre elicits a pattern of these responses within the stomach rather than separate relaxation, secretion and vasodilatation This hypothesis is based on the following considerations

1 There are very few efferent fibres in the vagus nerves For purposes of economy one would expect one fibre to serve more than one function especially since there seems to be an intimate interplay between the functions under discussion

2 Afferent stimulation of nerve fibres from chemo and mechanoreceptors in the gastrointestinal tract produces a vagally mediated response pattern with gastric hydrochloric acid and pepsin secretion and a prolonged relaxation of the stomach similar to that obtained in III—V (Harper Kidd and Scratcherd 1959)

3 Gastric secretion is accompanied by functional vasodilatation in the active mucosa (*e g* Wolf and Wolff 1943)

4 Gastric secretion induced by sham feeding is accompanied by gastric motor inhibition (Lorber Komarov and Shay 1950 Maung Pe Thein and Schofield 1959 Olbe and Jacobson 1963)

5 Topical stimulation of the cerebral cortex e g in the sigmoid gyrus elicits gastric secretion (Klopper 1954) Stimulation of the same area has been found to inhibit gastric motility (Eliasson 1952 Klopper 1954) Similar observations have been made on topical stimulation of hypothalamus (for ref see Eliasson 1960)

6 The above mentioned effects on the stomach relaxation, secretion and vasodilatation, can all be controlled across their entire range by an impulse frequency rate below 10 imp/sec (II and V)

7 The response pattern is physiologically meaningful during the cephalic phase of digestion appetite juice is secreted, this requires an increased blood flow, for at least the parietal cells of the stomach are among the most energy consuming cells of the body (Crane and Davis 1951) Furthermore a change in the myogenic tone of gastric muscle facilitates receptive relaxation At the same time the excitatory low threshold vagal fibres can effectively control the movements necessary for the mixing and emptying of the gastric contents In so far as the motility observed can be taken as an expression of these functions, it seems as if the latter are facilitated by the relaxation elicited by the high threshold vagal fibres

Summary

Various gastric responses to efferent vagal stimulation were studied in acute experiments on cats The following observations were made

1 The vagus nerves of the cat contain efferent nerve fibres of two types the excitation threshold of one differing from that of the other The low threshold group enhances gastric motility while the high threshold group enhances secretion of acid and pepsin, increases blood flow in the stomach, and produces marked relaxation of the corpus fundus region

2 The vagally elicited gastric motor inhibition is essentially a relaxation with a manifold increase in the volume of the stomach at very low transmural pressures This gastric relaxation is not adrenergic but elicited by some unknown mechanism It does not act by inhibiting the excitatory intramural ganglionic cells but exerts its action on the presumably myogenic activity of the gastric smooth muscles In these respects the vagal relaxing fibres differ strinkingly from the sympathetic inhibitory fibres

It seems probable that the gastric responses elicited by the high threshold fibres form a pattern of responses also under physiological conditions then being involved in the elicitation of the cephalic phase of digestion

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PREFACE

The subject of the present study was proposed in 1961 by Professor Olavi Eranko M D Ph D Head of the Department of Anatomy in the University of Helsinki It is part of a research project on the histochemistry of the nervous tissue directed by him

I wish to thank Professor Eranko most cordially for advice and encouragement from the preliminary studies to the preparation of the manuscript

Professor Unto Uotila M D read the manuscript and suggested significant alterations for which I am very grateful to him

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Helsinki April 1965

Ulla Soderholm

CONTENTS

INTRODUCTION

The motor neurones of the spinal cord have been the subject of several histological and biochemical studies Much of our knowledge of the nerve cells and their function has in fact been acquired from investigations dealing with the spinal cord (Bok 1928 Lowry 1957 Nachmansohn 1959 Giacobini 1959b Hydén 1960 Eccles 1964 Eccles and Schadé 1964) Some authors have also studied the characteristics of the motor neurones with the aid of histochemical methods and special attention has been paid to the cholinesterases of these cells and their synapses (see Koelle 1963)

However except in regard to the cholinesterases histochemical investigations have been few and often restricted to one or a few enzymes Usually studies on the motor neurones of the spinal cord have formed a minor part of general studies on the nervous system Studies concerning the non specific esterases and the oxidative enzymes of the spinal cord are especially few and the results have often been in conflict (Wolfgram and Rose 1959 Thomas and Pearse 1961 Nandy and Bourne 1964a b) A systematic investigation of the distribution of hydrolysing and oxidative enzymes therefore seemed called for

Still fewer investigations deal with the effect of nerve division on the enzyme activity in the motor neurones of the spinal cord Actually only acid phosphatase has been studied thoroughly in this respect (e g Colmant 1959 Barron and Sklar 1961 Barron and Tuncbay 1962 Kawai 1963) However histochemical studies on the behaviour of enzymes during the axon reaction may be expected to be fruitful because the function of the nerve cell is then radically changed and this causes variations in the components of the cell as was recently shown in the superior cervical ganglion by Harkonen (1964) By following the axon reaction with the aid of several different histochemical techniques it is possible to gain a better idea of the function of the nerve cell and its components

The aim of the present study has been to shed light on the activity and distribution of the carboxylic esterases phosphatases and tetrazolium reductases in the motor neurones of the anterior horn of the spinal cord of the rat and the changes caused by division of the lumbar nerves

EARLIER INVESTIGATIONS

CARBOXYLIC ESTERASES

Normal Neurones

Acetylcholinesterase

Histochemical Distribution A high acetylcholinesterase (AChE) activity has been demonstrated in the anterior horn of the spinal cord of many species Using acetylthiocholine as substrate in combination with various agents that inhibit AChE or non specific cholinesterase (ns ChE) activity several authors have observed a positive AChE reaction in the cytoplasm of the motor neurones of the cat (Koelle 1951 1955a Snell 1961) the rabbit the Rhesus monkey (Koelle 1955a) the bullfrog the common toad (Chacko and Cerf 1960) and the rat (Koelle 1954) Synaptic activity around these cells and the cell processes has been emphasized by Gerebtzoff (1953) and Koelle (1954 1955a) Eserine sensitive activity has been described in the motor neurones of the spinal cord when the substrate used demonstrates non specific esterase (ns E) activity (Ravin et al 1953 Csillik and Savay 1954)

Activity of Individual Cells With the Cartesian diver technique the motor neurones of the spinal cord of the rat have been divided into two groups one with low and one with high AChE activity (Giacobini and Holmstedt 1958) The cell body exhibits a high AChE activity the dendrites and the axon a somewhat lower activity and the nucleus 1/10—1/100 of the activity of other parts of the motor neurones of the spinal cord (Giacobini 1957 1959a b 1960)

Intracellular Distribution The distribution of histochemically demonstrable AChE activity in the neurones of the ciliary ganglion resembles that of the basophilic cytoplasmic material thus has been taken to indicate that AChE is synthesized in and associated with the endoplasmic reticulum (Fukuda and Koelle 1959 Fukuda 1959) This result agrees with those of biochemical studies in which the ChE has been found mainly in cytoplasmic particles especially in the membranous structures of the microsomal fraction of the brain of the rabbit (Nathan and Aprison 1955) and the rat (Aldridge and Johnson 1959 Hanzon and Toschi 1959 Holmstedt and Toschi 1959 Toschi 1959) Electron microscopically the AChE activity of the brain of the rat can be localized to the endoplasmic reticulum of the neurone cytoplasm (Torack and Barrnett 1962 Mori et al 1964)

Synaptic Distribution Histochemically demonstrable AChE activity in the ciliary ganglion of birds (Szentagothai et al 1954) and of the cat (Koelle and Koelle 1959 Koelle 1962) has been considered to be both pre and postsynaptic while it has been suggested to be mainly presynaptic in the hypoglossal nucleus of the rat (Schwarzacher 1958) Electron microscopically the synaptic activity in the medulla oblongata and the caudate nucleus of the rat has been reported to be located both pre and postsynaptically (Torack and Barrnett 1962 Lewis and Shute 1964) In subcellular fractions AChE has been shown biochemically to be concentrated in the synaptic membranes whereas acetylcholine and cholin acetylase are present in the synaptic vesicles (Whittaker and Gray 1962 De Robertis et al 1962 1963 Rodriquez De Lores Arnaiz and De Robertis 1962 Rodriquez De Lores Arnaiz 1964)

Activity of Nerve Fibres In nerve fibres of the anterior horn of the spinal cord and the sciatic nerve AChE activity has been demonstrated histochemically (Koelle 1951 1954 Tewari and Bourne 1960 Okinaka et al 1963) electron microscopically (Lewis and Shute 1964) and biochemically (Lubinska et al 1963)

Non specific Cholinesterase

Capillaries Ns ChE activity has been observed in the capillaries of the spinal cord of the rat (Koelle 1954 1955b Brightman and Albers 1959 Lewin 1961) and the cat (Koelle 1955a) and in the capillaries of the brain of the rat (Pepler and Pearse 1957 Teoharov 1962) However the ns ChE activity of the capillaries was considered to be an artifact by Gerebtzoff (1959) and no activity was detec ted in the capillaries of the spinal cord of the cat (Koelle 1951 Brightman and Albers 1959) the sheep and man (Brightman and Albers 1959) In the capillaries of the brain of the rabbit an AChE activity has been observed (Crook 1963)

Neurones and Neuroglia Whereas according to Brightman and Albers (1959) the only site of extraneuronal ns ChE in the spinal cord of the rat is the capillaries activity has been reported in the gliocytes and in a few motor neurones in the spinal cord of the rat (Koelle 1954) and in the gliocytes in the spinal cord and other nervous tissue of the cat (Koelle 1955a Brightman and Albers 1959) the rabbit the Rhesus monkey (Koelle 1955a) and the guinea pig (Gerebtzoff 1959) Ns ChE activity was observed by Harkonen (1964) in some nerve cells of the sympathetic ganglion of the rat where most cells were essentially devoid of such activity

Axonal Envelope In different regions of the human brain and spinal cord the white matter has been shown biochemically to exhibit more ns ChE activity than AChE activity (Ord and Thompson 1952 Cavanagh et al 1954) Ns ChE is considered to be a probable component of the Schwann cells and the suppor tive glia (Cavanagh et al 1954) Gerebtzoff (1959) considered that ns ChE activity is probably associated with the myelin sheath The neurokeratin network of the myelin sheath of the sciatic nerve of the rat also contains ns ChE accord ing to Tewari and Bourne (1960) However in a well controlled study it has

been shown histochemically that the myelin of the central and peripheral nervous systems is enzymatically inactive towards butyrylthiocholine (Adams et al 1963)

Non specific Esterases

Naphthyl Acetates In the motor neurones of the rat and the dog an esterase activity of varying intensity has been observed in the cytoplasm and nerve cell processes and the activity of this enzyme was recorded in spite of paraffin embedding (Hard and Fox 1951 Gomori and Chessick 1953 Pearse 1955) In these studies nothing is mentioned about the intracellular distribution of the activity Gossner (1958) paid attention to the activity of the nucleolus of the motor neurones of the human spinal cord Biochemically the highest ns E activity of the brain of the rat has been observed in the microsomal fraction (Aldridge and Johnson 1959 Sellinger and de Balbian Verster 1962)

Indoxyl Acetates Eserine resistant i e ns E activity has been observed in the neurones of the brain both in paraffin embedded and in frozen sections the latter either formol fixed or fresh (Pearse 1955 Pepler and Pearse 1957 Pearson and Grose 1959) In formol fixed frozen sections part of the esterase activity in the sections was due to diethyl p nitrophenyl phosphate (E600) resistant non specific esterase (E r ns E) (Pepler and Pearse 1957) Fresh frozen sections have been shown to exhibit a moderate ns E activity towards indoxyl acetate and 5 bromoindoxyl acetate in the cytoplasm the dendrites and the axon of the motor neurones of the spinal cord of the rat (Barrnett 1952 Pearse 1955) and paraffin embedding did not destroy the activity (Pearse 1955)

Naphthol AS Acetates The motor neurones have been reported to show activity towards naphthol AS acetates in the spinal cord in paraffin embedded sections of the rat (Gomori and Chessick 1953) in fresh postfixed sections of the guinea pig (Kumamoto and Bourne 1963) and in formol fixed sections of the rabbit (Wachstein et al 1961) In the spinal cord of the rabbit activity was mostly due to E-r ns E (Wachstein et al 1961) The lipofuscin pigments of ganglion cells of man have also been reported to show esterase activity (Gedigk and Bonthe 1956)

Thiolacetic Acid In the motor neurones of the spinal cord of the rabbit a diffuse staining has been reported in addition to staining in the cytoplasmic granules due to ns E activity part of which is due to E-r ns E (Wachstein et al 1961)

A reaction towards thiolacetic acid has been observed by electron microscopy in small cytoplasmic dense bodies in the neurones of the brain of the rat (Torack and Barrnett 1962) this activity was due to E r ns E apparently cathepsin C (Hess and Pearse 1958)

Starch Gel Electrophoresis

Starch gel electrophoresis has been applied to the study of esterase activity in brain tissue With α naphthyl acetate and naphthol AS acetate as substrates

9 bands were revealed (Barron et al 1961) of which the one with the lowest mobility was sensitive to eserine and reactive towards acetylthiocholine With 4-chloro-5 bromoindoxyl acetate as substrate the staining was confined to the three lowest ns E fractions

In the α naphthyl acetate zymogram of the brain of the rat Eranko et al (1962a b) found the ChE activity in the band nearest the origin they observed 3 bands of E r ns E activity and one band with the highest mobility due to E600 sensitive (E s) ns E activity The differences obtained with α naphthyl acetate and butyrate were marked enough to suggest the presence of truly substrate specific esterases

In an electrophoretic study of the human brain esterases the complexity of the esterolytic activity was subsequently confirmed (Barron et al 1963) Furthermore it has been demonstrated that readily soluble esterases entering the starch slab produce different zymograms from those obtained by liberating esterases firmly attached to tissues different substrates again showing different types of esterase activity (Eranko et al 1964)

The Axon Reaction

Cholinesterases

Biochemical Studies A 30% decrease in the ChE activity of the spinal cord of the cat was observed after bilateral deafferentation (Nachmansohn and Hoff 1944) A decrease of ChE activity has also been found in the sympathetic ganglion of the cat after preganglionic denervation (von Brucke 1937 Sawyer and Hollinshead 1945) After postganglionic denervation of the same ganglion of the cat and the rat a marked decrease in the AChE activity was observed (Sawyer and Hollinshead 1945 McLennan 1954)

Histochemical Studies Fifteen days after ligation of the sciatic nerve of the dog a loss of ChE activity from the neuropil and the neurones of the spinal cord was histochemically observed by Hard and Peterson (1949 1950) A reduction of the AChE activity has also been demonstrated in the motoneurones of the spinal cord of the bullfrog and the common toad (Chacko and Cerf 1960)

Four to 14 days after division of the hypoglossal nerve disappearance of the AChE activity was observed in the nerve cell cytoplasm and a decrease in the activity of the synapses and the nerve fibres of the hypoglossal nucleus of the rat (Schwarzacher 1958) Activity remaining at synapses was assumed to be presynaptically situated Gerebtzoff and Vandersmissen (1956) found a decrease in the AChE activity of the nerve cells of the dorsal nucleus of the vagus after cutting the vagus nerve

After section of the preganglionic trunk to the superior cervical ganglion practically all the AChE activity of the preganglionic fibres has been found to disappear whereas the activity of the ganglion cells remains unaffected in both the cat (Sawyer and Hollinshead 1945 Koelle 1951 Snell 1958 Taxi 1961 Fredricsson and Sjoqvist 1962 Holmstedt et al 1963) and the rat (Brown 1958 Harkonen 1964) After postganglionic denervation the ChE activity decreases in both the ganglion cells and the preganglionic fibres (Sawyer and Hollinshead 1945 Brown 1958 Fredricsson and Sjoqvist 1962, Harkonen 1964)

Non specific Esterases

After sciatic neurotomy of the guinea pig a decrease has been observed in the ns E activity of a number of nerve cells of the spinal ganglion (Kumamoto and Bourne 1963) After preganglionic denervation of the superior cervical ganglion of the rat no changes were observed in the ns E activity of the nerve cells while postganglionic denervation resulted in a reduction of E s ns E activity in them (Harkonen 1964)

In the motor neurones of the spinal cord of the guinea pig the ns E after division of the sciatic nerve showed a slightly decreased enzyme activity towards naphthol AS acetate in postfixed sections (Kumamoto and Bourne 1963)

PHOSPHATASES

Normal Neurones

Acid Phosphatase

A faint staining has been observed in the motor neurones of the spinal cord of the cat in paraffin embedded sections (Smith 1948) and those of the rabbit showed activity and a tendency to staining in granules (Samorajski and Fitz 1961) The cytoplasm of the motor neurones of the spinal cord of the guinea pig has been found to stain intensely and there was staining in the nucleus as well (LaVelle et al 1954) The nuclear staining however has been suggested to be an artifact due to diffusion (Eranko 1951 Holt 1959 Deane 1963)

A reaction in granules has been observed after formol fixation throughout the cytoplasm of the motor neurones of the spinal cord of the cat (Barron and Sklar 1961) and the rabbit (Barron and Tuncbay 1962) These granules were presumed to be the lysosomes (de Duve et al 1955 de Duve 1963) Sections of the spinal cord of the rat have been examined under the electron microscope for acid phosphatase and enzymatically active dense bodies have been found near the Golgi apparatus (Novikoff and Essner 1962)

Alkaline Phosphatase

When formol fixed frozen sections of the brain of the rat were used no activity in neurones o glia was detected by Becker et al (1960) or Fishman and Hayashi (1962) the only activity was localized in the capillaries In the spinal and trigeminal ganglia of the rat however Tewari and Bourne (1962 1964) reported a strong diffuse activity in the periphery of the nerve cells Nandy and Bourne (1963) also observed a reaction in the neurones of the brain of the rat, especially in the synaptic area in addition to intense activity of the nucleoli

In fresh sections of the spinal cord of the rat, the synaptic regions of the nerve cells have been reported to show alkaline phosphatase activity (Nandy and Bourne 1963) with glycerophosphate a reaction was observed in the nerve cell cytoplasm and cell processes while these structures did not show activity towards naphthol AS phosphates Activity has also been reported in the nerve cell processes of the spinal cord of the rabbit but none was observed in the nerve cell cytoplasm (Samorajski and Fitz 1961)

In the spinal cord the alkaline phosphatase activity has been found to be high in the terminal arteries and the capillaries (Bannister and Romanul 1963)

Adenosine Triphosphatase

Using different methods Wawrzyniak (1963) studied the distribution of adenosine triphosphatase (ATPase) in the motoneurones of the spinal cord of the guinea pig in the cytoplasm there were three zones with different intensities of reaction in the perinuclear space the enzyme activity was high in the zone near the axon the activity was weakest and the peripheral zone of the perikaryon showed a moderately variable reaction The activity was situated in the mito chondria in the cell membranes and in the system of endoplasmic membranes in addition to the nucleus and nucleolus

In the brain of the rat, the ATPase activity has been shown to be confined to the membranes of the Purkinje cells and other neurones in addition to the capillaries (Becker et al 1960 Novikoff et al 1961) However an earlier report by Naidoo and Pratt (1951) claimed that the cytoplasm of the nerve cells of the brain of the rat shows activity in addition to staining of the nuclei of the nerve cells which was later confirmed (Naidoo 1962)

In the nerve cells of the spinal and trigeminal ganglia and the Purkinje cells of the cerebellum of the rat the ATPase activity has been reported to be situated in the cytoplasm and to change its distribution cyclically sometimes being in the cell periphery sometimes around the nucleus or widely distributed through out the cytoplasm the cycle also being reflected in the nucleus and the nucleolus (Tewari and Bourne 1962 1963a b) Activity located in the nucleolus has been especially claimed by Sandler and Bourne (1962)

Electron Microscopic Studies With the electron microscope it has been observed that the ATPase of the brain of the rat is situated in the interspace between the plasma membranes of the neurones and glial dendrites adjacent to the neuro nal cell body the proximal axon and the synaptic terminals and also in the Golgi apparatus of the nerve cells (Torack and Barrnett 1963)

In the nerve cells of the vestibular nucleus of the rabbit the main ATPase activity has been detected in the glial cells smaller amounts being present in the neurones and inside the neurone at the cell membrane as determined bio chemically from single cells and parts of cells (Cummins and Hyden 1962)

Thiamine Pyrophosphatase

In the cells of many organs of the rat, e g in the Purkinje cells in formol fixed sections the thiamine pyrophosphatase activity has been localized to the Golgi apparatus the stained Golgi lamellae were extensive and reached into the dendrites (Novikoff and Goldfischer 1961)

Barron and Tuncbay (1962) have reported that the motor neurones of the spinal cord of the rabbit show activity towards thiamine pyrophosphate The site of the activity resembled the configuration of the Golgi apparatus the lamellar system filling the cytoplasm and extending into the dendritic processes but not into the axons As a result of electron microscopic studies the thiamine pyrophosphatase activity has been reported to be located in the Golgi apparatus in the motor neurones of the spinal cord of the rat (Novikoff and Essner 1962)

The Axon Reaction

Acid Phosphatase

Biochemically no change in activity was found by Fieschi and Soriani (1959) in the spinal cord of the guinea pig after bilateral neurotomy of the sciatic nerve while Samorajski and Fitz (1961) observed an increase of activity on both sides of the spinal cord of the rabbit after unilateral sectioning of the nerve

An increase of acid phosphatase activity has been demonstrated histochemically in the motor neurones of the spinal cord after division of the sciatic nerve of the monkey (Bodian and Mellors 1945) the cat (Smith 1948 Lassek and Bueker 1947) the domestic fowl the frog (Bueker et al 1949) the bullfrog (Cerf and Chacko 1958) the rabbit (Barron and Tuncbay 1962) the guinea pig (Kawai 1963) and the rat (Colmant 1959) whereas no change was detected in the rabbit by Bueker et al (1949) or Samorajski and Fitz (1961)

An increase of the activity of the neurones of the facial nucleus has also been observed after section of the facial nerve (Coimbra and Tavares 1964) The cells of the stellate ganglion and the dorsal nucleus of the vagus have been observed to show increased activity after axotomy (Smith and Luttrell 1947)

The motor neurones of the cervical part of the spinal cord and those of the hypoglossal nucleus were found to exhibit increased acid phosphatase activity after plexotomy or hypoglossal neurectomy of the cat (Barron and Sklar 1961) the lysosomes exhibiting the acid phosphatase activity were enlarged, and the cytoplasm was packed with them Increased acid phosphatase activity was also seen in the motor neurones of the spinal cord of the rabbit, and was reported to be due to an increase in the number and enzymatic activity of the lysosomes (Barron and Tuncbay 1962)

Alkaline Phosphatase

A decrease of alkaline phosphatase activity was found biochemically by Fieschi and Soriani (1959) and Samorajski and Fitz (1961) in the spinal cord of the guinea pig and the rabbit after division of the sciatic nerve No histochemical changes were observed by Samorajski and Fitz (1961)

Adenosine Triphosphatase

A reduction of calcium activated ATPase has been detected biochemically in the spinal cord of the guinea pig after section of the sciatic nerves (Fieschi and Soriani 1959)

Thiamine Pyrophosphatase

A reduction of the staining of the Golgi lamellae of the motor neurones of the spinal cord has been observed after division of the sciatic nerve of the rabbit (Barron and Tuncbay 1962) the thiamine pyrophosphatase active reticulum in chromatolytic neurones was displaced to the extreme periphery of the swollen perikaryon or disappeared In the spinal cord of the rat, the Golgi lamellae of the motor neurones have also been shown to become thinner and less active after sectioning of the motor nerve (Novikoff and Essner 1962)

Normal Neurones

The NAD... ...H tetrazolium reductase activities of the spinal cord ... man have been studied by Friede (1959) Samorajski (1961) Friede and Fleming (1962) and Nandy and ... investigations the activity was demonstrable both in the ... intervening tissue the neuropil the motor neu rone ... relatively strong activity while that of the intervening neuropil ...

In ... the spinal cord of the rat a relatively high activity ... DP linked tetrazolium reductases has been shown, ... and diffusely distributed in the dendrites or the glial ... 961) Activity towards α glycerophosphate was weak ... nal cord the satellite cells showing a strong reaction ... oquinone (menadione) was used as an intermediate ... and Pearse 1961 Thomas and Pearse 1961) According ... (1959) the activity of several tetrazolium reductases was ... l cells of the spinal cord of the rat and the cat.

... nes of the spinal cord of the rat the cat and the monkey ... succinate tetrazolium reductase has been reported by Friede ... (1961) In the intervening neuropil of the rat and the cat, ... ion as in the motor neurones (Friede 1959)

The Axon Reaction

Biochemical Studies A decrease of succinic dehydrogenase activity has been biochemically demonstrated in the spinal cord after division of the sciatic nerve of the Rhesus monkey (Howe and Flexner 1947) and the guinea pig (Kumamoto and Bourne 1963)

In the sympathetic ganglion of the rat, an increase in the activities of lactic and glucose 6 phosphate dehydrogenases after postganglionic denervation has been reported by Harkonen (1964)

Histochemical Studies Succinate and NADPH tetrazolium reductases have been found to diminish conspicuously in the motor neurones of the spinal cord of the rat after division of the sciatic nerve while the activity of NADH tetrazolium reductase diminished to a lesser degree (Friede 1959) Under similar conditions the motor neurones of the spinal cord of the guinea pig have been reported to show a reduction of succinate tetrazolium reductase activity while apparently no changes were observed in regard to the intensity of NADH tetrazolium reductase (Kumamoto and Bourne 1963)

The succinate tetrazolium reductase activity in the neurones of the spinal ganglion of the rat has been shown to increase after division of the sciatic nerve (Klein 1960) The nerve cells of the facial nucleus show an increase in the NADPH and NADP linked glucose 6 phosphate tetrazolium reductase activity after

sectioning of the facial nerve of the guinea pig (Kreutzberg 1963) and an increase in the levels of a number of other tetrazolium reductases in those of the rabbit (Fischer and Malik 1964) An increase in the activities of all the tetrazolium reductases studied was similarly noted histochemically in the sympathetic ganglion of the rat after axotomy (Harkonen 1964)

PROBLEMS OF THE PRESENT STUDY

As is apparent from the review of the literature the enzyme histochemistry of the nervous system has been the subject of many conflicting observations and many features of obvious interest have not been investigated at all Therefore the present study was undertaken in order to investigate systematically the distribution of a large variety of enzymes in the anterior horn of the spinal cord The following questions appeared especially interesting

1 Can different types of esterases be demonstrated in the spinal cord?
2. What are the electrophoretic properties of these enzymes?
3 How does nerve division affect the different types of esterases of the neurones and the synapses?
4 Is there any hydrolytic enzyme activity in the cytoplasmic granules of the motor nerve cells and are these granules affected by the axon reaction?
5 What are the distributions of alkaline phosphatase and adenosine triphosphatase in the anterior horn and how are they affected by the axon reaction?
6 Are there differences in the distribution of different tetrazolium reductases in the motor nerve cells and the neuropil, and how does axon division affect the activity of tetrazolium reductases?

MATERIAL AND METHODS

Normal Material

The material comprised about 200 normal rats of the Sprague Dawley strain. The ages of the rats varied from 3 to 6 months For histochemical studies only male rats were used but for electrophoretic studies both males and females No sexual differences were observed

The rats were killed by decapitation under mild ether anaesthesia The lumbar spinal cord was immediately dissected free and the lower part of the lumbar enlargement was used for the study

Fresh frozen sections, sections postfixed after sectioning and sections cut from fixed tissue were used. To obtain fresh sections the spinal cords were frozen fresh with solid carbon dioxide ice on a specimen holder with a drop of 10 % gelatine solution sectioned at 10 μ in a cryostat and allowed to thaw on cover slips at room temperature These sections were then either used fresh or were postfixed for 20 min at room temperature in formol-calcium or in formol dextran. The formol-calcium fixative consisted of one volume of neutralized 35 formaldehyde 6 volumes of 2 calcium chloride and 3 volumes of distilled water and the formol-dextran fixative of one volume of neutralized 35 formaldehyde one volume of 1 calcium chloride and 8 volumes of dextran solution (Macrodex, Pharmacia Sweden) Fixation of fresh tissue was carried out in formol-calcium for 4 hours at room temperature After a quick rinse in distilled water frozen sections were cut at 10 μ with a freezing microtome and the sections were then treated free floating

Experimental Procedure

To study the axon reaction of the neurones of the anterior horn the right lumbar nerves of 76 male rats 2 to 4 months of age were divided The left side served as control

Ether anaesthesia was used. The right lumbar nerves were dissected free within the psoas muscle and the anterior rami of the third fourth fifth and nearly always the sixth lumbar nerves were cut with sharp scissors 2 to 3 mm from the intervertebral foramina The nerve stumps were left among the muscles The wound was sutured with silk. The rats generally recovered well after the operation However they were never able to stand on the right leg afterwards The divided nerves were the main trunks to the femoral, obturator and sciatic nerves

The number of rats killed at different times after the operation can be seen from Table 1

Table 1

Anterior horns studied at different times after nerve division.

Days after nerve division	Number of rats
1—3	8
4—7	15
8—10	11
11—20	23
21—50	6
51—100	7
101—182	6

CARBOXYLIC ESTERASES

Sections

Cholinesterases

Acetyl- and butyrylcholinesterase. For the demonstration of ChEs the method originally described by Koelle (1950, 1951) was used, as modified by Gomori (1952b). Sections were preincubated in a buffer solution with or without inhibitor for 30 min. at 37° C, and subsequently incubated for 3 hours at 37° C in a substrate medium containing acetyl- or butyrylthiocholine iodide and the inhibitor in the same concentration as in the preincubation solution.

The following inhibitors were used: 1:5-bis-(4-allyldimethylammoniumphenyl) pentan-3-one dibromide (284C51) in a concentration of 10^{-5}M to inhibit AChE activity and tetraisopropylpyrophosphoramide (iso-OMPA) in a concentration of 10^{-5}M to inhibit ns. ChE activity (Holmstedt 1957a,b; Pepler and Pearse 1957).

Controls were made by using an incubation solution without any substrate or by using both 10^{-5} M 284C51 and 10^{-5} M iso-OMPA together or 10^{-5}M eserine alone to inhibit ChEs (Gomori 1952a). These controls were always negative.

Non-specific Esterases

Fresh or unfixed cryostat sections from fresh material mounted on slides or free floating sections from fixed tissue were used. The sections were preincubated in a buffer solution at the same pH as the incubation solution with or without an inhibitor for 30 min. at room temperature.

AChE was inhibited with 10^{-5} M or 10^{-4}M 284C51, ns. ChE with 10^{-5} M iso-OMPA and both ChEs with 10^{-5} M eserine. The esterases were divided into two groups according to their behaviour towards 10^{-5} M E600 (Eränkö et al. 1962c, 1964).

α-Naphthyl acetate and butyrate, 4-chloro-5-bromoindoxyl acetate, and naphthol AS-D acetate were employed as substrates. The incubation solution contained the same concentration of the inhibitor as the preincubation solution.

Controls were made by incubation in a solution without either the substrate or the Blue BB Salt. They were always negative. The activity of fresh sections was destroyed by heating to 80° C in distilled water for one min., that of fixed sections dewaxed in 5 min. at 80° C.

α-Naphthyl Acetate and Butyrate The incubation at room temperature required from 4 to 5 min when α-naphthyl acetate was used and from 20 to 30 min when α-naphthyl butyrate was used as substrate The Blue RR Salt was used as coupler and the incubation solution was prepared according to Eränkö et al (1962c)

4-Chloro-5 Bromoindoxyl Acetate The incubation solution was prepared according to Holt (1958) The incubation required 30 to 60 min. for fixed sections and 1 to 2 hours for fresh sections

Naphthol AS D Acetate The incubation took 45 min at room temperature in the substrate solution made according to Planteydt (1961) with Blue RR Salt as coupler

Starch Gel Electrophoresis

For electrophoretic study the tissue was immediately frozen on carbon dioxide ice The white matter was subsequently dissected away and the posterior horn removed, so as to get reasonably pure anterior horns for homogenization The pieces of anterior horns were weighed and homogenized in a glass homogenizer with 1 ml of distilled water for 200 mg of tissue The homogenate was frozen and thawed five times and centrifuged for 30 min at 25 000 x g Twenty μl of the supernatant was pipetted on a piece of filter paper (Whatman No 1) and this sample was inserted into the gel The gel was made of Connaught starch in borate buffer according to Eränkö et al (1962b) A voltage gradient of 6 V/cm was applied for 3 hours at room temperature.

After electrophoresis the 9-mm-thick slabs were sliced into three slices These slices were preincubated for 30 min in a preincubation solution with or without an inhibitor and then immersed in the substrate solution. The inhibitors used were the same as employed for sections

To study the effect of formalin on the zymogram, one slice was immersed in the formol calcium fixative for 30 min. washed for 30 min. in distilled water and preincubated and incubated as described The neighbouring slice from the same slab was incubated in calcium chloride solution without formalin washed and stained

Efforts were made to characterize lyo-and desmo-enzymes by starch gel electrophoresis according to the procedure reported by Eränkö et al (1964) The anterior horns were frozen on a specimen holder and sections 100 μ thick were cut and mounted on slides in a cryostat The sections were allowed to dry at room temperature and 0 5 ml of distilled water was pipetted on them After 1 min the water was collected. This water contained the soluble esterase fraction (the lyo-fraction) The sections were then washed in distilled water for 30 min to remove the rest of the lyo-enzymes frozen and thawed five times removed from the slide and homogenized, then again frozen and thawed five times and centrifuged for 30 min at 25 000 x g The desmo-enzymes solubilized in this way were collected in the supernatant

Except for small variations in the substrate solutions and longer incubation times the methods were the same as those used for the sections In the acetyl and butyrylthiocholine method, the incubation time was prolonged to 24 to 48 hours The Blue RR Salt concentration of the α-naphthyl acetate and butyrate method was reduced (Eränkö et al. 1962b) and the incubation times were prolonged to 30 min and 1 hour respectively The concentration of the redox buffer of the 4-chloro-5-bromoindoxyl acetate method was decreased (Barron et al. 1963 Härkönen 1964) In this and the naphthol AS-D acetate method the incubation time was about 4 hours at 37 C

Controls were made as with sections using incubation solutions without the substrates They were always observed to be negative

PHOSPHATASES

Ordinary fresh postfixed and fixed sections were used except when ATPase was to be demonstrated. Then fresh sections postfixed for 5 min. at —3 C in formol-calcium were used (Wachstein et al. 1962)

The incubation solutions of acid and alkaline phosphatases were made according to Gomori (1952b) with sodium α and β-glycerophosphate as substrates Nucleoside phosphatases were demonstrated with the substrates adenosine 5-triphosphate and thiamine pyrophosphate and the methods of Wachstein and Meisel (1957) and Novikoff and Goldfischer (1961) respectively The incubation times were 30 min. at 37 C for all of these.

Controls were made by incubating in incubation solution without the substrates The controls sometimes showed staining of the nucleus or the nucleous like that seen in the corresponding substrate sections Such staining was considered non specific (Holt 1959) Controls of acid phosphatase were made by incubation with the substrate and 0 01 M sodium fluoride (Newman et al. 1950) These were negative

TETRAZOLIUM REDUCTASES

NADH and NADPH-tetrazolium reductases were demonstrated by the method of Scarpelli et al. (1958) with reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) as substrates The incubation time was 20 min at 37 C.

NAD^+ and $NADP^+$ linked tetrazolium reductases were incubated for 30 min at 37 C in an incubation medium according to Hess et al. (1958) The substrates used were malic acid sodium lactate isocitrate β-hydroxybutyrate glutamate α glycerophosphate and potassium glucose 6-phosphate. Glucose-6-phosphate-tetrazolium reductase was demonstrated as $NADP^+$ linked the others as NAD^+ linked In the present study nitro-BT was used as final electron acceptor

α-Glycerophosphate menadione tetrazolium reductase was demonstrated as the NAD^+ linked enzyme with menadione instead of NAD^+ (Wattenberg and Leong 1960 Wattenberg 1961)

A mixture of sodium succinate in phosphate buffer and nitro-BT in aqueous solution was used to demonstrate succinate tetrazolium reductase according to Nachlas et al. (1957) The effect of phenazine methosulphate was studied.

To obtain more information about the intracellular distribution sections were fixed in cold acetone for 5 min. (Novikoff et al. 1960) or a piece of material was fixed in block for 3 or 5 min. at 4 C in each of 0 7 1.3 and 2 % formaldehyde in Hanks balanced salt solution (Walker and Seligman 1963)

Controls were made by incubation in a medium without the substrate the trace of endogenous substrates having been washed away by incubating the sections for 5 min in phosphate buffer The NAD^+ $NADP^+$ and menadione linked tetrazolium reductases were also controlled by incubating them in a solution without NAD^+ $NADP^+$ and menadione These controls were all negative. Other sections were inactivated by heating to 80 C for 3 min. in distilled water

RESULTS

CARBOXYLIC ESTERASES

Normal Neurones

Sections

In the description of the results the esterases are divided into four categories according to the classification adopted in our laboratory (Eranko et al 1962c, 1964 Esila 1963 Harkonen 1964) The ChE activity is divided into AChE and ns ChE activity using 284C51 and iso-OMPA as inhibitors The ns Es are divided into E s ns E and E-r ns E using E600

The intensities of the esterase activities of the main structures in the anterior horn of the spinal cord are presented in Tables II—IV as obtained with different substrates and substrate inhibitor combinations The intensities are expressed with the signs +++ ++ + ± and — which should be read intense moderate weak very weak and no activity respectively Table II shows the activity in fresh sections Table III in fresh sections postfixed for 20 min in formol-dextran at room temperature (»postfixed sections») and Table IV in sections cut from tissues fixed for 4 hours at room temperature in formol-calcium (»fixed sections»)

Acetylcholinesterase

Acetylthiocholine Effect of Fixation The best results in the demonstration of AChE activity were obtained with acetylthiocholine and free floating sections from fixed tissue (Table IV) Longer fixation up to 24 hours affected the activity very little In fresh sections (Table II) a less intense reaction was obtained after equally long incubation (3 hours) and probably diffusion artifacts made it difficult to identify the positive structures The synapses looked like a thick membrane around the cells However no essential differences in activity were observed between fresh and fixed sections Postfixed sections (Table III) showed almost as intense an activity as fixed sections (Table IV) but they too suffered from diffusion artifacts The behaviour towards the different inhibitors was the same irrespective of fixation

Effect of Inhibitors To inhibit the ns ChE activity 10^{-6}M or 10^{-7}M iso-OMPA was used Both concentrations inhibited the activity of the capillaries which was the only site of ns ChE activity in the anterior horn of the spinal cord

Table II

Esterases in Fresh Sections

The intensities of the esterase activities of the main structures in the anterior horn of the spinal cord in fresh sections as obtained with different substrates and substrate inhibitor combinations

Substrates and Substrate Inhibitor Combinations	Nerve Cell Cytoplasm		Synapses	Nerve Fibres	Blood Vessels	Peri cytes
	Back ground	Granules				
acetylthiocholine	+	−	++	+	+	−
10^{-4}M iso-OMPA	+	−	++	+	−	−
10^{-5}M 284C51	−	−	−	−	+	−
10^{-5}M eserine	−	−	−	−	−	−
10^{-5}M E600	−	−	−	−	−	−
butyrylthiocholine	−	−	−	−	+	−
10^{-4}M iso OMPA	−	−	−	−	−	−
10^{-5}M 284C51	−	−	−	−	+	−
10^{-5}M eserine	−	−	−	−	−	−
10^{-5}M E600	−	−	−	−	−	−
α naphthyl acetate	+++	−	−	+	−	−
10^{-5}M iso OMPA	+++	−	−	+	−	−
10^{-5}M 284C51	+++	−	−	−	−	−
10^{-5}M eserine	+++	−	−	−	−	−
10^{-5}M E600	−	−	−	−	−	−
α naphthyl butyrate	++	−	−	−	−	−
10^{-5}M iso-OMPA	++	−	−	−	−	−
10^{-5}M 284C51	++	−	−	−	−	−
10^{-5}M eserine	++	−	−	−	−	−
10^{-5}M E600	−	−	−	−	−	−
4-chloro-5 bromo-indoxyl acetate	++	−	−	+	−	−
10^{-5}M iso-OMPA	++	−	−	+	−	−
10^{-5}M 284C51	++	−	−	−	−	−
10^{-5}M eserine	++	−	−	−	−	−
10^{-5}M E600	−	−	−	−	−	−
naphthol AS D acetate	+	−	−	−	−	−
10^{-5}M iso-OMPA	+	−	−	−	−	−
10^{-5}M 284C51	+	−	−	−	−	−
10^{-5}M eserine	+	−	−	−	−	−
10^{-5}M E600	+	−	−	−	−	−

Table III

Esterases in Postfixed Sections

The intensities of the esterase activities of the main structures in the anterior horn of the spinal cord in postfixed sections as obtained with different substrates and substrate inhibitor combinations

Substrates and Substrate Inhibitor Combinations	Nerve Cell Cytoplasm		Synapses	Nerve Fibres	Blood Vessels	Pericytes
	Background	Granules				
acetylthiocholine	++	−	++	++	++	−
10^{-4}M iso-OMPA	++	−	++	++	−	−
10^{-5}M 284C51	−	−	−	−	++	−
10^{-5}M eserine	−	−	−	−	−	−
10^{-5}M E600	−	−	−	−	−	−
butyrylthiocholine	−	−	−	−	++	−
10^{-4}M iso-OMPA	−	−	−	−	−	−
10^{-5}M 284C51	−	−	−	−	−	−
10^{-5}M eserine	−	−	−	−	−	−
10^{-5}M E600	−	−	−	−	−	−
α naphthyl acetate	++	++	+++	+	++	+
10^{-5}M iso OMPA	++	++	+++	+	−	+
10^{-5}M 284C51	+	++	−	−	++	+
10^{-5}M eserine	+	++	−	−	−	+
10^{-5}M E600	±	++	−	−	−	+
α naphthyl butyrate	+	+	−	−	++	+
10^{-5}M iso OMPA	+	+	−	−	−	+
10^{-5}M 284C51	+	+	−	−	++	+
10^{-5}M eserine	+	+	−	−	−	+
10^{-5}M E600	±	+	−	−	−	+
4-chloro 5 bromo indoxyl acetate	++	+	++	+	++	+
10^{-5}M iso OMPA	++	+	++	+	−	+
10^{-5}M 284C51	+	+	−	−	++	+
10^{-5}M eserine	+	+	−	−	−	+
10^{-5}M E600	±	+	−	−	−	+
naphthol AS D acetate	+	++	−	−	−	+
10^{-5}M iso OMPA	+	++	−	−	−	+
10^{-5}M 284C51	+	++	−	−	−	+
10^{-5}M eserine	+	++	−	−	−	+
10^{-5}M E600	+	++	−	−	−	+

Table IV

Esterases in Fixed Sections

The intensities of the esterase activities of the main structures in the anterior horn of the spinal cord in fixed sections as obtained with different substrates and substrate inhibitor combinations.

Substrates and Substrate Inhibitor Combinations	Nerve Cell Cytoplasm		Synapses	Nerve Fibres	Blood Vessels	Pericytes
	Background	Granules				
acetylthiocholine	++	−	+++	+++	++	−
10^{-5}M iso-OMPA	++	−	+++	+++	−	−
10^{-5}M 284C51	−	−	−	−	++	−
10^{-5}M eserine	−	−	−	−	−	−
10^{-5}M E600	−	−	−	−	−	−
butyrylthiocholine	−	−	−	−	++	−
10^{-5}M iso-OMPA	−	−	−	−	−	−
10^{-5}M 284C51	−	−	−	−	++	−
10^{-5}M eserine	−	−	−	−	−	−
10^{-5}M E600	−	−	−	−	−	−
α-naphthyl acetate	++	+++	++	−	−	++
10^{-5}M iso-OMPA	++	+++	++	−	−	++
10^{-5}M 284C51	−	+++	−	−	−	++
10^{-5}M eserine	−	+++	−	−	−	++
10^{-5}M E600	±	+++	−	−	−	++
α-naphthyl butyrate	−	++	−	−	−	−
10^{-5}M iso-OMPA	−	++	−	−	−	−
10^{-5}M 284C51	−	++	−	−	−	−
10^{-5}M eserine	−	++	−	−	−	−
10^{-5}M E600	±	++	−	−	−	−
4-chloro-5-bromo-indoxyl acetate	++	++	++	−	−	++
10^{-5}M iso-OMPA	++	++	++	−	−	++
10^{-5}M 284C51	−	++	−	−	−	++
10^{-5}M eserine	−	++	−	−	−	++
10^{-5}M E600	−	++	−	−	−	++
naphthol AS-D acetate	−	+++	−	−	−	+++
10^{-5}M iso-OMPA	−	+++	−	−	−	+++
10^{-5}M 284C51	−	+++	−	−	−	+++
10^{-5}M eserine	−	+++	−	−	−	+++
10^{-5}M E600	−	+++	−	−	−	+++

Even 10^{-7}M iso-OMPA however reduced the AChE activity and therefore it was not generally used 10^{-8}M 284C51 inhibited the AChE activity totally

Distribution The strongest activity was observed in the synapses around the motor neurones (Table IV Figs 1 14 and 20) The synapses were also distributed around the nerve cell processes and in the neuropil often in clumps The thin nerve fibres connected with the synapses also exhibited an intense activity The cytoplasm of the motor neurones showed less strong activity varying from weak to moderate No relation between the size of the cell and the cytoplasmic activity was noted The nucleus was always negative In the neuropil there were many fibres with strong activity some of which were seen to continue into the white matter

Other Substrates Butyrylthiocholine did not show any AChE activity since 10^{-5}M 284C51 did not have any effect, and the activity towards it was totally inhibited by 10^{-7}M iso-OMPA Apparently neither α naphthyl butyrate nor naphthol AS-D acetate with Blue RR Salt as coupler demonstrated AChE, because 284C51 did not reduce the intensity of the reaction obtained with these substrates (Tables II III and IV)

In fixed and postfixed sections α naphthyl acetate and 4-chloro-5 bromoindoxyl acetate gave a reaction which was partly inhibited by 284C51 a concentration of 10^{-4}M was required with α naphthyl acetate and 10^{-5}M was enough with 4-chloro-5-bromoindoxyl acetate (Tables III and IV) (The inhibition was like that in Figs 7 and 8 in which the effect of eserine is illustrated No difference was observed between the effects of 284C51 and eserine except for the activity of ns ChE of the capillaries) The distribution of the AChE activity was the same as that described when acetylthiocholine was used as substrate

In fresh sections nearly all the activity demonstrable in the neuropil with α naphthyl acetate or 4-chloro-5-bromoindoxyl acetate was inhibited by 10^{-5}M 284C51 or 10^{-5}M eserine (Table II) No inhibition was observed in the motor neurone cytoplasm. Any AChE activity that might have been demonstrable with these substrates in the motor neurone was masked by a strong ns E activity (Figs 4 and 5)

Non specific Cholinesterase

Butyrylthiocholine Whether fresh or fixed sections were used ns ChE activity was observed in the capillaries (Tables II III and IV Fig 2) This activity was inhibited by 10^{-7}M iso-OMPA and it was not affected by 10^{-5}M 284C51 Even if the incubation time was prolonged to 24 hours no activity towards butyrylthiocholine was found in the motor neurones the synapses or the nerve fibres

Other Substrates Acetylthiocholine showed the same reaction in the capillaries and this reaction was totally inhibited with 10^{-} M iso-OMPA (Tables II III and IV) α Naphthyl acetate, butyrate and 4-chloro-5-bromoindoxyl acetate in postfixed sections showed a moderate reaction associated with the capillaries

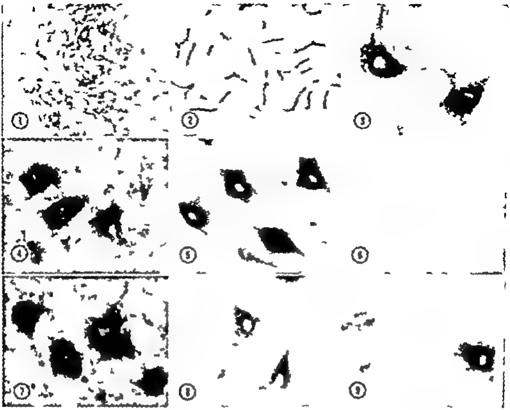


Fig 1 Cholinesterases in a fixed section Acetylthiocholine no inhibitor x 80

Fig 2 Non specific cholinesterase in a fixed section Butyrylthiocholine and 10^{-5}M 284C51 x 80

Fig 3 Esterases in a fixed section Naphthol AS-D acetate no inhibitor x 200

Fig 4 Esterases in a fresh section α Naphthyl acetate no inhibitor x 200

Fig 5 E600-sensitive non specific esterase in a fresh section. α Naphthyl acetate and 10^{-5}M eserine x 200

Fig 6 E600-resistant non specific esterase in a fresh section No reaction visible α Naphthyl acetate and 10^{-5}M E600 x 200

Fig 7 Esterases in a fixed section α Naphthyl acetate no inhibitor x 200

Fig 8 Non specific esterases in a fixed section α Naphthyl acetate and 10^{-5}M eserine x 200

Fig 9 E600-resistant non specific esterase in a fixed section α Naphthyl acetate and 10^{-5}M E600 x 200

and sensitive to 10^{-5}M iso-OMPA (Table III) In fixed sections the reaction was faint (Table IV) and in fresh sections it was not observed (Table II) Naphthol AS D acetate did not exhibit any activity in the capillaries (Tables II III and IV)

E600 sensitive Non specific Esterase

α Naphthyl Aceta e The best way to demonstrate E s ns E activity was to use this substrate and Blue RR Salt as applied to fresh sections together with 10^{-5}M eserine to inhibit ChE activity The activity thus demonstrated was totally in hibited in fresh sections by 10^{-5}M E600 (Table II Figs 5 and 6)

Distribution The reaction was almost exclusively limited to the motor neu rones (Fig 5) in which the cytoplasm was intensely stained either evenly or in patches

The nucleus was always negative A faint activity was seen in the neuropil perhaps due to nerve cell processes but the sections being fresh diffusion interfered with the localization.

Effect of Fixation When α naphthyl acetate was used as substrate with 10^{-5}M eserine postfixation for 10 min at room temperature reduced the even distribu tion of the activity in the motor neurones as compared with fresh sections but some of the remaining activity was resistant to 10^{-5}M E600 This activity not present in fresh sections was associated with cytoplasmic granules Postfixation for 2 hours resulted in a great reduction of the whole staining of the cell, the reaction being restricted to small granules and the reaction was altogether resis- tant to 10^{-5}M E600 Postfixation for 20 to 30 min revealed both E s and E r ns E activity (Table III) and 20 min fixation was generally used

When the fixation was carried out before sectioning the penetration of the fixative into the deeper parts of the tissue was uneven However in sections from blocks fixed for 4 hours at room temperature the E r ns E was strong compared with the weak or absent E s ns E activity (Table IV Figs 8 and 9)

Other Substrates In fresh sections the ns E reaction obtained with α naphthyl butyrate or 4-chloro-5 bromoindoxyl acetate as substrates was totally abolished by 10^{-5}M E600 Naphthol AS D acetate did not show any E s ns E (Table II)

E600-resistant Non specific Esterase

α Naphthyl acetate and naphthol AS-D acetate gave similar results (Figs 3 and 9) Fixed sections were used other esterase activity being inhibited with 10^{-5}M E600 Not even 10^{-3}M E600 inhibited this reaction

Distribution A very faint even background staining was observed in the motor neurones besides a reaction located in the cytoplasmic granules The granules were often clustered round the nucleus or a limited area of the cell. Cells without granules were rare The nucleus was negative

In the neuropil, no activity was observed except for dark staining of occasional cells round the blood vessels (pericytes?) (Fig 24)

In postfixed sections the same staining was seen though weaker (Table III)

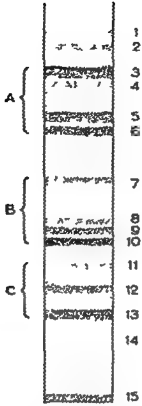


Diagram I Schematic illustration of the electrophoretic results obtained with different esterase substrates Further data on the different bands are presented in Table V

Other Substrates In fixed and postfixed sections α naphthyl butyrate and 4 chloro-5 bromoindoxyl acetate showed the same reaction as that obtained with α naphthyl acetate and naphthol AS D acetate (Tables III and IV) In fresh sections no such activity was seen (Table II) except when naphthol AS D acetate was used This substrate demonstrated a faint diffuse activity in the motor neurones which was totally resistant to 10^{-5}M E600 (Table II)

Starch Gel Electrophoresis

The results obtained with all the substrates employed are schematically summarized in Diagram I The bands are numbered No 1 being the fastest band and No 15 the band at the origin (Webb 1964) Three areas denoted A B and C, exhibited a diffuse reaction extending over several bands in the upper middle and lower parts of the zymogram respectively

In Table V the reactions obtained in each band with different substrates are shown The reaction intensities are denoted with the signs +++ ++ + and — signifying the presence of intense moderate weak and no activity The effects of the inhibitors are indirectly shown with the aid of footnotes indicating the type of activity present. For example column α naphthyl acetate Band No 12 which is marked ++ and is seen from the footnotes to contain E r ns E activity means that the activity remaining in the presence of eserine was not

... reduced by $10^{-5}M$ (or $10^{-4}M$) E600. The diffuse area C, which contains ... extends over Band 12 (Diagram I) and because the activity of this area ... inhibited by eserine, the intensity of the reaction in Band No. 12 is also ... reduced, although this is not considered to be due to the ChE activity ... band.

At least 3 and usually 10 zymograms were prepared for each substrate inhibitor combination, and the bands of these zymograms were compared. The pattern was very complex. Attention is therefore paid to some features only.

Cholinesterases

Except for acetyl- and butyrylthiocholine, α-naphthyl acetate was the only substrate which showed activity inhibited by $10^{-5}M$ eserine, i.e. ChE activity. Such activity was shown in the diffuse Areas B and C. These areas were at the same level as the corresponding areas in the acetylthiocholine zymogram (Fig. 10). The reaction of Areas B and C was inhibited by $10^{-5}M$ 284C51 or $10^{-5}M$ eserine while $10^{-4}M$ iso-OMPA caused only slight reduction of the activity. Some activity was also seen at the same levels in the butyrylthiocholine zymogram, though it was very weak. Thus the ChE activity of Areas B and C was chiefly due to AChE, although a slight activity of ns ChE was detected at about the same level.

Non-specific Esterases

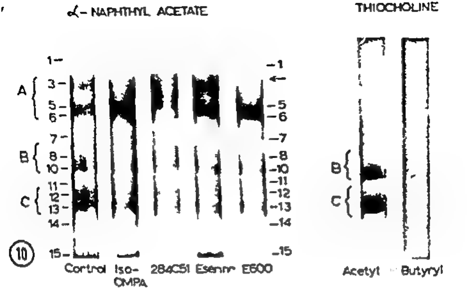
Most of the bands in the α-naphthyl acetate zymogram were due to ns Es and except for Bands 3 and 15, which were due to E-s ns E, they exhibited E-r ns E activity. If any E-s ns E was also present in these bands the E-r ns E activity was strong enough to mask it (Fig. 10).

Most of the bands demonstrated with the other substrates were also represented in the α-naphthyl acetate zymogram (Table V, Figs. 10 and 11). However with naphthol AS-D acetate one band (No. 2) was demonstrated at a level at which there was no corresponding activity in the α-naphthyl acetate zymogram. α-Naphthyl butyrate revealed another such band (No. 4). Naphthol AS-D acetate and α-naphthyl butyrate both demonstrated Band 9, which was not visualized in the α-naphthyl acetate zymogram. Peculiarly enough, this band was due to E-r ns E activity in the naphthol AS-D acetate zymogram, and E-s ns E activity in the α-naphthyl butyrate zymogram.

Even if other bands demonstrated with the other substrates were at the same level as a corresponding band in the α-naphthyl acetate zymogram, they were not necessarily due to the same type of esterase. Thus all the bands in the α-naphthyl butyrate zymogram were due to E-s ns E activity and all the bands in the naphthol AS-D acetate and 4-chloro-5-bromoindoxyl acetate zymograms were due to E-r ns E activity.

Effect of Formalin

Zymograms fixed in formol-calcium before staining showed a weakening of the activity of the bands demonstrable with α-naphthyl acetate and butyrate. When naphthol AS-D acetate was used, however, an increase of the activity was noted (Fig. 12).



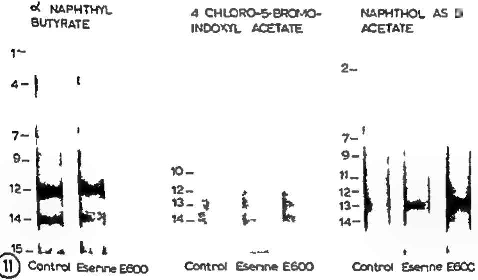
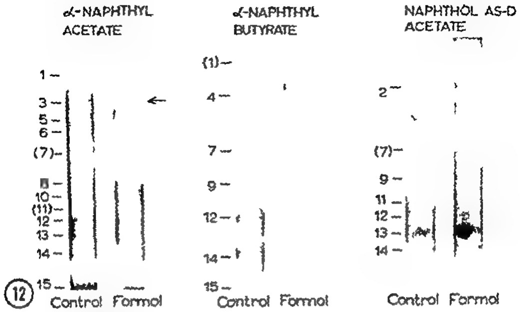


Fig 10 Esterases in zymograms obtained with α naphthyl acetate acetyl and butyrylthiocholine Arrow shows lack of activity in the presence of 10 M E600

Fig 11 Effect of 10^{-5}M eserine and 10^{-5}M E600 on esterase zymograms obtained with different substrates



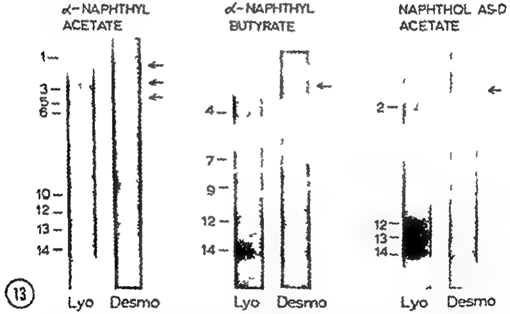


Fig. 12 Effect of formalin on esterase zymograms. Arrow shows diminution of activity at the level of the E600-sensitive non specific esterase band.

Fig. 13 Comparison of zymograms obtained with lyo- and desmo-esterases. Arrows show activity of desmo-zymograms not seen in lyo- or homogenate zymograms.

Lyo- and Desmo-Esterases

The zymograms demonstrating the lyo- and desmo-esterases with α naphthyl acetate butyrate and naphthol AS D acetate as substrates differed from each other (Fig 13) In the lyo-zymogram the bands resembled those of the homogenate zymogram however in the lyo-zymogram Band 3 due to E s ns E activity was stronger than Bands 5 and 6 due to E-r ns E activity with α naphthyl acetate as substrate In the desmo zymogram Bands 3 5 and 6 were lacking but there was a new moderately active band between the levels of Bands 1 and 3 and two less active bands with lower mobility None of these three bands had any corresponding activity in the lyo or homogenate zymograms In the α naphthyl acetate lyo-zymogram all the bands were due to E r ns E activity except for Band 3 In the desmo-zymogram the activity of all these bands almost vanished with 10^{-4}M E600 The very faint trace of activity sometimes left was considered to be due to contamination of the lyo-fraction

In the α naphthyl butyrate and naphthol AS D acetate zymograms the main difference between the lyo- and desmo-esterases were also observed in the upper part of the zymogram With α naphthyl butyrate as substrate Band 4 in the lyo-zymogram was strong This band was not seen in the desmo-zymogram whereas with higher mobility there was a moderately reactive band All the bands demonstrable with α naphthyl butyrate were sensitive to 10^{-4}M E600

With naphthol AS D acetate as substrate Band 2 was moderately stained in the lyo-zymogram It was not demonstrable in the desmo zymogram At a level above this there was a very faint activity in the desmo-zymogram which did not correspond to any band in the lyo or homogenate zymograms All the bands demonstrable with naphthol AS D acetate were due to E r ns E activity

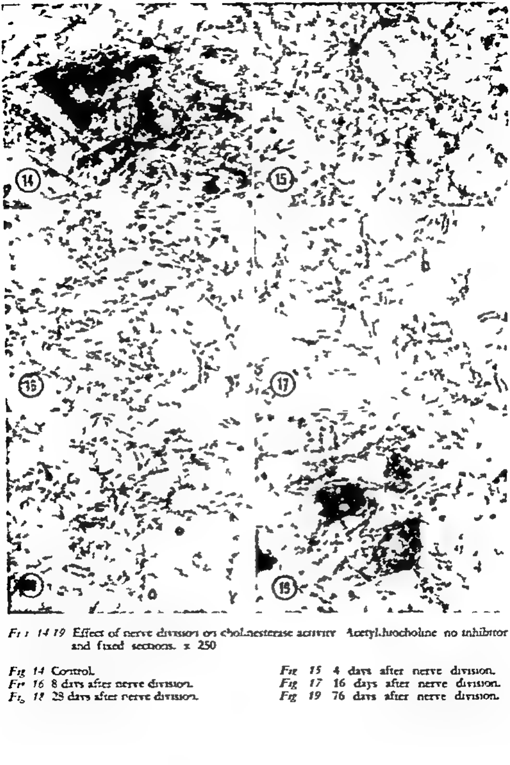
The Axon Reaction

Acetylcholinesterase

In judging the effect of the nerve division anterior horns in the same section were compared with each others the left horn serving as a control to the right denervated horn The left horns did not show noticeable differences from those in entirely untreated controls

Changes were never observed in all the cells and the changes in the individual cells varied with time The present description is based on the mean appearance of the cells

The first changes in the AChE activity were observed about 3 days after nerve division The staining of the motor neurones was less intense (Fig 15) About 8 days after nerve division the staining of the chromatolytic cells was faint (Fig 16) The activity seemed to disappear especially from the periphery of the cytoplasm and the number of active synapses was also much decreased About 8 to 30 days after the operation the activity of the motor neurones remained faint (Figs 17, 18 and 21) The number of active synapses was further diminished but positive synapses were seen at all times Likewise the number of active fibres was reduced About 30 to 70 days after the operation the activity of the motor neurones increased



Figs. 14-19 Effect of nerve division on cholinesterase activity. Acetylthiocholine no inhibitor and fixed sections. x 250

Fig. 14 Control.
Fig. 15 4 days after nerve division.
Fig. 16 8 days after nerve division.
Fig. 17 16 days after nerve division.
Fig. 18 28 days after nerve division.
Fig. 19 76 days after nerve division.

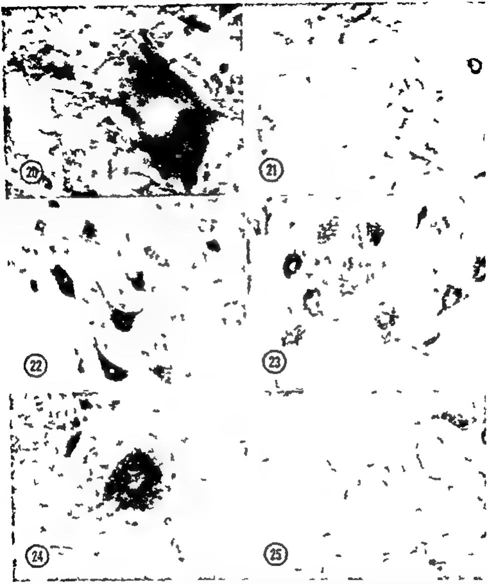


Fig. 20 Cholinesterases in a fixed section. Acetylthiocholine, no inhibitor. Control. *Fig.* 21 The same reaction 28 days after nerve division. x 500

Fig. 22 E600-sensitive non specific esterase in a fresh section. α Naphthyl acetate and 10^{-4}M eserine. Control. *Fig.* 23 The same reaction 12 days after nerve division. x 100

Fig. 24 E600-resistant non specific esterase in a fixed section. α Naphthyl acetate and 10^{-4}M E600. Control. *Fig.* 25 The same reaction 58 days after nerve division. x 250

Nearly all the cells showed normal cytoplasmic activity 70 to 90 days after the operation (Fig 19) However, even now there were cells with very faint activity About 180 days after nerve division no differences from normal could be detected any longer The number of active synapses increased apparently to normal 70 to 180 days after the operation, and so also did that of the nerve fibres

Non specific Cholinesterase

No change was observed in the ns ChE activity which was limited to the capillaries

E600 sensitive Non specific Esterase

About 5 days after nerve division the enzyme activity began to decrease The maximum decrease though weak was observed 12 days after nerve division and normalization began after as little as 30 days and was complete 50 days after the operation

Because this type of activity was selectively demonstrable only in fresh sections the changes in the intracellular location were difficult to record The remaining activity was evenly distributed in vacuolized cells (Fig 23)

E600 resistant Non specific Esterase

This enzyme selectively demonstrable in fixed sections showed a decrease in the motor neurones 5 days after nerve division The staining of the granules weakened About 8 to 60 days after denervation many cells without any staining in the granules were seen in others the reaction was weakened (Fig 25) Thereafter the activity increased again and more granules became visible About 70 days after the operation the reaction was nearly normal

PHOSPHATASES

Normal Neurones

Acid Phosphatase

The only site of activity was in the motor neurones In fixed sections small granules stained deeply brown in the cytoplasm (Fig 26) They were distributed fairly evenly throughout the whole nerve cell cytoplasm and extended into the cell processes

When fresh sections were used the staining was evenly distributed in the cytoplasm in postfixed sections it was both diffuse and in granules

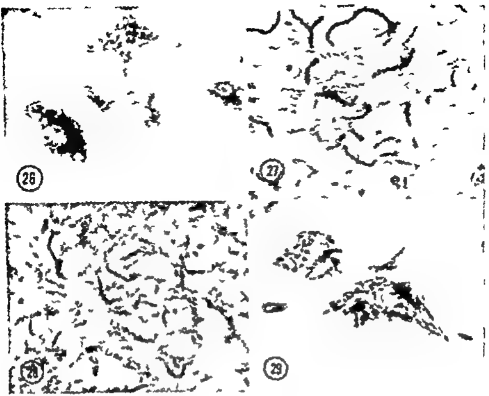


Fig 26 Acid phosphatase in a fixed section. x 250

Fig 27 Alkaline phosphatase in a fixed section. x 100

Fig 28 Adenosine triphosphatase in a postfixed section x 100

Fig 29 Thiamine pyrophosphatase in a fixed section x 300

Alkaline Phosphatase

In fresh and fixed sections the only positive site observed was in the capillaries (Fig 27)

Adenosine Triphosphatase

No activity was observed in the cytoplasm of the motor neurones whether fixed postfixed or fresh sections were used The fresh sections suffered from diffusion artifacts

Activity was strongest in the capillaries Around the motor neurones in fixed and postfixed sections there was activity apparently situated in the cell membrane of the neurone In the neuropil there was activity which was difficult to localize (Fig 28)

Thiamin Pyrophosphatase

In fixed sections the activity was visualized in the cytoplasm of the motor neurones as bands and strings of beads situated concentrically around the nucleus filling the whole cytoplasm and reaching to the nerve cell processes (Fig 29) This activity was apparently in the Golgi apparatus

In fresh and postfixed sections the activity of the Golgi apparatus was more diffuse.

A strong activity was seen in the capillaries but these also stained with glycerophosphates as substrates in this incubation medium. With glycerophosphates as substrates there was no staining of the motor neurones

The Axon Reaction

Acid Phosphatase

About 5 days after nerve division the activity of this enzyme normally present in cytoplasmic granules in the motor neurones began to increase. The number of granules increased but so did the intensity of the reaction in the individual granules too Between 8 and 50 days after the operation the middle of the cell showed an accumulation of highly active granules (Fig 31) The histochemical appearance was almost normal 70 days after the operation

Alkaline Phosphatase

No change was observed in the activity which was limited to the capillaries

Adenosine Triphosphatase

The motor nerve cell cytoplasm which did not normally show any activity did not become positive after nerve division However 5 days after denervation short bands of strong activity showed at the cell membrane and these bands became longer From about the 7th day on, the whole cell was surrounded by a strong activity at the membrane of the cell (Fig 33) This activity was still demonstrable 40 days after the operation Thereafter it began to decrease Most of the cells looked normal on the 70th day but 130 days after the operation cells with a strongly positive membrane were still found

Thiamin Pyrophosphatase

The reaction of the Golgi apparatus began to decrease 7 days after the operation. The Golgi structures appeared narrower than normal, and the reaction intensity was lower (Fig 35) About 10 to 40 days after nerve division there were large areas especially in the middle of the cell, entirely devoid of any of the activity which normally filled the whole cell. The Golgi structure was almost normal 70 days after the operation.

Fig 30 Acid phosphatase in a fixed section Control x 250

Fig 31 The same reaction 41 days after nerve division x 250

Fig 32 Adenosine triphosphatase in a postfixed section Control x 100

Fig 33 The same reaction 17 days after nerve division x 100

Fig 34 Thiamine pyrophosphatase in a fixed section Control x 500

Fig 35 The same reaction [illegible] days after nerve division x 500

TETRAZOLIUM REDUCTASES

Normal Neurones

NADH, NADPH, and NAD+ and NADP+ linked Tetrazolium Reductases

The enzymes demonstrated showed an almost identical distribution The strongest activity was found in the motor neurones (Figs 36—40) A faint staining was also seen in the walls of the blood vessels No activity was observed in the glial cells but there was a faint activity in the neuropil which seemed to be situated in the nerve cell processes No special activity was seen around the nerve cells at the sites of the synapses

In the motor neurones the staining was both diffuse and particulate The diffuse staining was evenly distributed all over the cell the particulate staining was localized in small granules the mitochondria In some cells these granules were distributed evenly in the cytoplasm in others they were present only around the nucleus or in a limited area of the cell In some cells no positive granules were seen at all The nucleus was never positive

The staining intensity obtained with different substrates differed NADH tetrazolium reductase always showed the strongest reaction β hydroxybutyrate and especially α glycerophosphate being the substrates giving the faintest reaction NADH malate and isocitrate tetrazolium reductases seemed to give rise chiefly to staining in the granules the others mainly led to diffuse staining

α Glycerophosphate Menadione and Succinate Tetrazolium Reductases

The distribution of these enzymes differed totally from those mentioned above Most of the activity was in the neuropil and it was difficult to localize it to any structures (Fig 41) From the cell membrane the neuropil was evenly stained and no special activity was observed at the site of the synapses In the cytoplasm of the motor nerve cells a few active granules were seen in the periphery of the cell or around the nucleus The nucleus did not stain Phenazine methosulphate speeded up the reaction with succinate as substrate but the distribution of the activity did not change

The Axon Reaction

NADH NADPH and NAD+ and NADP linked Tetrazolium Reductases

Changes in the activities of these enzymes were small but still detectable An increase in the reaction intensity of the motor neurone was observed 5 to 8 days after nerve division The maximum of this increase lasted from about 12 to 50 days after the operation Both the diffuse and the particulate staining seemed to be affected the activity being concentrated in the middle of the chromatolytic cell (Figs 43 45 and 47) About 70 days after nerve division the activity was normal again

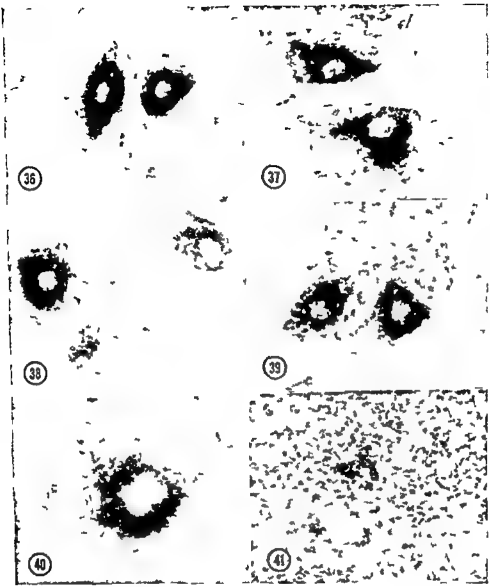


Fig 36 NADH tetrazolium reductase in a fresh section x. 250

Fig 37 NADPH tetrazolium reductase in a fresh section. x 250

Fig 38 Lactate tetrazolium reductase in a fresh section. x 250

Fig 39 Glucose-6-phosphate-tetrazolium reductase in a fresh section. x 250

Fig 40 Malate tetrazolium reductase in a fresh section x 500

Fig 41 Succinate tetrazolium reductase in a fresh section x 500

TETRAZOLIUM REDUCTASES

Normal Neurones

NADH NADPH and NAD^+- and $NADP^+$ linked Tetrazolium Reductases

The enzymes demonstrated showed an almost identical distribution The strongest activity was found in the motor neurones (Figs 36—40) A faint staining was also seen in the walls of the blood vessels No activity was observed in the glial cells but there was a faint activity in the neuropil which seemed to be situated in the nerve cell processes No special activity was seen around the nerve cells at the sites of the synapses

In the motor neurones the staining was both diffuse and particulate The diffuse staining was evenly distributed all over the cell the particulate staining was localized in small granules the mitochondria In some cells these granules were distributed evenly in the cytoplasm in others they were present only around the nucleus or in a limited area of the cell In some cells no positive granules were seen at all The nucleus was never positive

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The increase of the NADH tetrazolium reductase activity was more evident than that of the other enzymes and that of α glycerophosphate was faintest. The changes in the activities of the other enzymes due to nerve division were about equal.

α Glycerophosphate Menadione and Succinate Tetrazolium Reductases

No clear changes were observed after nerve division

DISCUSSION

Different Types of Esterase Activity. It is well known that the esterases form a large group of enzymes which differ from each other in regard to preference for substrates, behaviour towards inhibitors, sensitivity to formalin, electrophoretic mobility etc. (Chessick 1954, Gomori 1955, Allen et al. 1958, Eränkö et al. 1962a,b,c, 1964).

In the present study two different types of ns Es were detected with the aid of 10⁻ M E600 in the motor neurones of the spinal cord. Only E-s ns E activity was seen in fresh sections while fixed sections showed E-r ns E activity almost exclusively. In fresh sections E-r ns E activity was lacking, but even a short postfixation made it histochemically demonstrable. It therefore seems evident that E-r ns E is very soluble and diffuses into the incubation medium. E-s ns E has a strong affinity for the tissue and is separable only with difficulty from the section. According to Nachlas et al. (1956) and Hannibal and Nachlas (1959) the lyo-enzymes or component of the esterases extractable when the sections are immersed in water are responsible for 60 %, of the total esterase activity. The lyo-component diffused into formol, too, but not so readily as into water.

In fixed sections E-s ns E activity was lacking or weak. It was perhaps partly masked by E-r ns E activity, but because of differences in the distribution of the activity this could not be the sole explanation. It seemed evident that the E-s ns E activity was inactivated by formalin, as has been reported by Hannibal and Nachlas (1959).

In the present study E-s ns E activity was distributed diffusely or somewhat patchily in the neurone cytoplasm, whereas that of the E-r ns E was distributed in the granules of the cells with a very faint even background staining.

The differences in the solubilities of E-s and E-r ns Es were also evident in starch gel electrophoresis. In the lyo- and homogenate zymograms most of the demonstrable activity was due to E-r ns E, the readily soluble part of the esterase activity, whereas the desmo-bands were due to E-s ns E activity. The latter part of the activity was difficult to extract from the sections: it was demonstrable in unfixed sections and solubilized only after intensive homogenization and many freezings and thawings. The E-s ns E demonstrable in the homogenate zymograms was sensitive to formalin as it was in sections.

Substrate Specificity. In both the sections and the zymograms α-naphthyl acetate was hydrolysed by E-s and E-r ns E activity, while naphthol AS-D acetate was hydrolysed only by E-r ns E activity. The two other substrates, α-naphthyl butyrate and 4-chloro-5-bromoindoxyl acetate, gave both E-s and E-r ns E

reactions in the sections while in the zymograms α naphthyl butyrate was hydrolysed by E s ns E activity and 4-chloro-5 bromoindoxyl acetate by E r ns E activity Although the lacking activity was perhaps masked by the stronger one different types of esterases clearly preferred different substrates

In earlier investigations however E r ns E activity has been revealed by zymograms with α naphthyl butyrate as substrate e g in the sympathetic ganglion of the rat (Harkönen 1964) and in the retina of several different mammals (Esila 1963) However no such activity was detected in the brain of the rat and the hamster (Eranko et al 1962b) or the spinal ganglion of the rat (Kokko to be published) 4-Chloro-5 bromoindoxyl acetate also showed some E s ns E activity in the zymograms of both the sympathetic and the spinal ganglion of the rat (Harkonen 1964 Kokko to be published)

The present study and the above mentioned investigations indicate that there are several types of esterases in the neural tissues Although the species and the methods of these investigations have been the same differences have been detected between these different nervous tissues — the motor neurones of the spinal cord the retina the sympathetic ganglion and the spinal ganglion These differences may be only quantitative because small amounts are not detected with the methods used

Enzyme Activity of Granules in Motor Neurones The periodic acid Schiff positive glycolipoprotein granules the lipofuscin granules and autofluorescent granules of such structures as the motor neurones of the spinal cord closely resemble in size number shape and topographical distribution the particles which give a strong reaction for acid phosphatase (Koenig 1962 1963 1964 Kawai 1963 Sharma 1964) The granules showing the acid phosphatase and/or E r ns E activity may be the lipofuscin granules (Gedigk and Bontke 1956) the lysosomes (de Duve et al 1955 Wachstein and Meisel 1961 Novikoff and Essner 1962 Koenig 1962 de Duve 1963 Holt 1963 Novikoff 1963 Goldfischer et al 1964) or the dense bodies (Torack and Barrnett 1963)

However in the present study the granules showing the acid phosphatase activity differed in location from those showing E r ns E activity The granules containing acid phosphatase were distributed throughout the cell cytoplasm reaching to the cell processes while the granules containing E r ns E were concentrated around the nucleus or in a limited area often in the middle of the cell This suggests the presence of different types of granules in the cells or different enzyme activities in these granules

In the present study nerve division caused a clear decrease in the number of E r ns E-positive granules while the number of acid phosphatase positive granules increased The number of lysosomes in the motor neurones after axotomy as judged from light microscopic observations of acid phosphatase preparations has been reported to increase by Barron and Sklar (1961) as well as by Novikoff and Essner (1962) Electron micrographs revealed an increase in the number of the dense bodies during the axon reaction (Hudson et al 1961) The observations of an increase in the acid phosphatase positive granules and of a decrease in the E-r ns E positive granules of the present study affords further evidence of the presence of two different types of granules and suggests differences in the function and properties of the two types of granules

the nerve cells In the neuropil however the succinate-tetrazolium reductase activity was intense whereas that of malate and isocitrate was faint Biochemically using single cell determinations the glial cells of the spinal ganglion and the vestibular nucleus of the rabbit have been observed to utilize succinate and pyruvate more that the nerve cells (Hydén et al 1958 Hydén and Pigon 1960 Hamberger 1961)

Because succinate tetrazolium reductase has been considered to be associated especially with the mitochondria (Pearse 1957 Scarpelli and Pearse 1958 Friede and Pax 1961 Rodriquez De Lores Arnaiz and De Robertis 1962) this rather exceptional distribution in the present study in regard to succinate-tetrazolium reductase was astonishing In order to compare the number and distribution of the mitochondria in the neurone cytoplasm and the neuropil, electron micrographs of the anterior horn of the spinal cord of the rat were made (unpublished) The abundance of mitochondria in these structures was found to be the same However the mitochondria in the cytoplasm of the motor neurones were larger and less electron dense than those of the neuropil The difference in the appearance of the mitochondria might also explain the differences in the content or histochemical staining of this enzyme

CONCLUSIONS

The results obtained in the present study have clearly indicated that several enzymes are present in different sites in the motor nerve cells and that they respond in different ways to axon division The following main conclusions which answer the questions presented earlier in this paper would seem to be justified

1 Esterase activity in fresh and fixed sections is due to many different enzymes which can be differentiated according to behaviour towards different substrates and inhibitors and towards fixation

2 The esterases can be separated by electrophoresis into several bands with widely differing substrate inhibitor characteristics

3 Axon division causes a reduction of the acetylcholinesterase activity of the motor nerve cells and of the synapses attached to them as well as a reduction in the activity of the non specific esterases

4 E600 resistant non specific esterase and acid phosphatase are both mainly situated in the cytoplasmic granules of the motor nerve cells but each of these two enzymes is partly present in distinct granules During the axon reaction the number of E600-resistant non specific esterase positive granules diminishes and the number of acid phosphatase positive granules increases

5 Alkaline phosphatase activity is located only in the capillaries of the anterior horn of the spinal cord and no change in the activity in response to nerve division is seen Adenosine triphosphatase activity is found at the cell membrane in addition to the capillaries the cell membrane activity clearly increases during the axon reaction

6 The activies of α glycerophosphate menadione and succinate tetrazolium reductases in the motor nerve cells are weak as compared with those of the neuropil and the axon reaction has no effect on them Other tetrazolium reductases show activity mainly in the cytoplasm of the motor nerve cells and a clear increase in their activity is seen during the axon reaction

SUMMARY

The present histochemical study deals with the activity and distribution of carboxylic esterases phosphatases and tetrazolium reductases in normal motor neurones of the spinal cord of the rat In order also to study the effect of neurotomy the lumbar nerves were divided unilaterally and the above mentioned enzymes were examined in the axotomized neurones 1 to 180 days after nerve division

Carboxylic Esterases The carboxylic esterases were demonstrated using as substrates acetyl and butyrylthiocholine, α naphthyl acetate and butyrate as well as 4 chloro 5 bromoindoxyl acetate and naphthol AS D acetate With the aid of specific inhibitors i e 284C51 iso OMPA eserine and E600, the group of carboxylic esterases was separated into acetylcholinesterase (AChE) non specific cholinesterase (ns ChE) and E600 sensitive and E600-resistant non specific esterases (E s and E r ns Es) Starch gel electrophoresis of the esterases was also performed

The motor neurones exhibited acetylcholinesterase activity In the perikaryon of the cell the reaction was moderate while the synapses and nerve fibres showed intense activity During the axon reaction a decrease was observed in the cytoplasmic activity 3 to 70 days after the operation while a decrease in the synaptic activity took place somewhat later 5 to 130 days after nerve division

Non specific cholinesterase activity was demonstrable in the capillaries only E600-sensitive non specific esterase activity was seen in all the motor neurones Since this type of activity was readily demonstrable in fresh sections it seemed evident that it was firmly attached to the tissue Part of the E600 sensitive non specific esterase activity proved sensitive to fixation in formalin Relatively little of this activity was demonstrable in the homogenate zymograms while the *desmo zymogram* mainly *showed* this type of activity During the axon reaction the E600 sensitive non specific esterase activity was somewhat diminished The reaction was normalized within about 50 days after the operation

E600 resistant non specific esterase activity was observed only in fixed sections The activity was associated with cytoplasmic granules around the nucleus or a limited area of the cell From fresh sections this type of activity was dissolved in the incubation medium but even a short postfixation in formalin immobilized the enzyme The lyo- and homogenate zymograms mainly exhibited this type of activity During the axon reaction the E600-resistant non specific esterase activity of the cytoplasmic granules disappeared or clearly diminished It was normal again about 70 days after nerve division

Phosphatases Acid phosphatase was located in cytoplasmic granules the granules being distributed throughout the cell The intensity of the enzyme reaction in individual granules and the number of granules increased during the axon reaction The distribution of acid phosphatase and E600-resistant non specific esterase positive granules was different and these granules behaved in a different way during the axon reaction

Alkaline phosphatase was demonstrated in the capillaries only Adenosine triphosphatase was apparently associated with the cell membranes of the neurones and this activity was clearly increased as a result of nerve division Thiamine pyrophosphatase was found in the Golgi apparatus and the activity was reduced during the axon reaction

Tetrazolium Reductases The NAD+ linked malate lactate isocitrate glutamate β hydroxybutyrate and α glycerophosphate tetrazolium reductases the NADP+ linked glucose 6-phosphate tetrazolium reductase as well as NADH and NADPH tetrazolium reductases were demonstrated using nitro-BT The distribution of these enzymes was closely similar and the main activity was in the perikarya of the motor neurones After nerve division the activity of these tetrazolium reductases increased to appear normal again about 70 days after nerve division

α Glycerophosphate menadione and succinate tetrazolium reductases in contrast to the above mentioned enzymes were mainly confined to the intercellular tissue i e the neuropil Only a few reactive granules were seen in the cytoplasm of the motor neurones and no change due to nerve division was detected

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An experimental and comparative study of its anatomical and functional organization in some mammals, including the presence of adrenaline and chromaffin cells in these organs

By

NILS O SJÖSTRAND

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According to the classical concept of the anatomical organization of the mammalian autonomic nervous system, postganglionic sympathetic fibres emerge from cell bodies located rather far away from the effector organs. Thus the fibres of the thoracolumbar, i.e. sympathetic outflow, have their synaptic relays in the ganglia of the sympathetic chain, the *paravertebral ganglia*, or in the ganglia of the abdominal plexa, the *prevertebral ganglia*. Only very few preganglionic sympathetic fibres have been assumed to end on ganglia closely related to the target organs, so-called *terminal ganglia*. This pattern of distribution of synaptic relays seems to be applicable for the major adrenergic part of the sympathetic outflow as well as for the minor cholinergic one.

In contrast to this arrangement, the preganglionic fibres of the cholinergic parasympathetic, i.e. craniosacral outflow, reach out to peripheral ganglia *(terminal ganglia)* lying within or in the close vicinity of the effector organs.

Although Langley and Anderson suggested as early as 1895 that part of the sympathetic fibres to the pelvic organs relay in ganglia close to the target organs, the general opinion has been that the accessory male genital organs are supplied by postganglionic fibres emanating from the inferior mesenteric ganglion and running down the hypogastric nerves.

The present investigation was prompted by the observations on the guinea pig that the motor response of the vas deferens to hypogastric nerve stimulation was inhibited by ganglionic blocking agents and that section and degeneration of the sympathetic nerves to the internal genital organs did not overtly reduce their noradrenaline content, thus indicating the existence of a peripheral synapse located in or near the effector organ and belonging to the adrenergic innervation of these organs. They would then, in contrast to the general pattern of sympathetic innervation, be innervated mainly by "short" adrenergic neurons instead of "long" ones. A further reason for the investigation was given by the large amounts of noradrenaline present in the internal accessory male genital organs, indicating a very rich adrenergic innervation or the presence of chromaffin cells in these tissues. Because of the great variability of these organs among mammals the investigation was extended to species other than the guinea pig.

The results will be presented in three sections. In the first part (chapter

IV) the effect of ganglionic blocking agents on the motor response of the isolated guinea-pig vas deferens to hypogastric nerve stimulation is described The second part (chapter V) deals with the catecholamine content of the accessory male genital organs of different species and the effect of sympathetic denervation upon this content In the third section (chapter VI) the cellular localization of the catecholamines is considered

Preliminary reports of some of the results included in the present study have been published (Sjöstrand 1962a, b) The histo-chemical work constituting chapter VI has previously been included in two separate reports (Falck, Owman and Sjöstrand 1965, Owman and Sjöstrand 1965)

CHAPTER I

HISTORICAL SURVEY

The first investigator who studied the innervation of the internal male genital organs from a physiological point of view seems to have been Budge* (1858) He found that stimulation of the inferior mesenteric ganglion and the hypogastric nerve produced contraction of the vas deferens of the rabbit Contraction was also obtained by stimulating the lumbar communicants to the ganglion The investigation of Budge was pursued further by Loeb (1866), who also described contraction of the seminal vesicle upon hypogastric nerve stimulation Remy (1886) observed contraction of the guinea pig vas deferens upon stimulation of the inferior mesenteric ganglion Sherrington (1892) found that stimulation of the twelfth thoracic and the first, second and third lumbar nerves especially the latter two of the rhesus monkey and the third and fourth lumbar nerves of the cat caused contraction of the *vasa deferentia*

Most of our knowledge in this field we owe however to the extensive studies of Langley and Anderson (1894 1895 and 1896) They concluded that in the rabbit the third fourth fifth and sometimes the second lumbar nerves send efferent fibres to the vas deferens and the seminal vesicle A similar lumbar origin was found for the fibres to the cat vas deferens The fibres were found to run to the inferior mesenteric ganglion From the ganglion motor fibres proceeded by the hypogastric nerves to the internal genital organs Stimulation of the hypogastric nerve caused contraction of the vas deferens and the seminal vesicle but no effect was obtained when the pelvic nerve or any other nerve in this region was stimulated With the use of nicotine application they suggested that at least a part of the preganglionic fibres passed the inferior mesenteric ganglion to run down the hypogastric nerve and join more peripheral nerve cells Nerve cells in the vicinity of the vas deferens and the prostate were also described In contrast to this however the overwhelming majority of fibres in the hypogastric trunk of the cat were found to be nonmyelinated Langley and Anderson further

* The investigators previous to Langley and Anderson do not use the terms inferior mesenteric ganglion and hypogastric nerve Since it is clearly understood from their descriptions that they mean these structures these terms have been used throughout this review

found that atropine, even in extremely high doses, had no effect on the response to hypogastric nerve stimulation Akutso (1903), in a report which seems to have been left out of account, described contraction of the vas deferens and the seminal vesicle of the guinea pig upon stimulation of the hypogastric nerve He suggested that the preganglionic fibres to the organs relayed in the inferior mesenteric ganglion and in peripheral ganglia located at the base of the seminal vesicle

Ganglion cells in the nerve plexus of the prostate were probably first demonstrated by Johannes Muller (1835) Later several investigators reported on nerve cells in the nerve plexa of this and other internal genital organs of different species (Leydig 1850, Reinert 1869, Timofeew 1894, Disselhorst 1897, 1904 G Muller 1904 and other early investigators The existence of nerve cells in the distal part of the hypogastric nerve has also been confirmed by recent investigators (Merrilees, Burnstock and Holman 1963, Vanov and Vogt 1963)) These organs were also found to possess a rather dense network of nerve fibres, Leydig 1850 and later Disselhorst 1904 pointed out the unusual richness of nerves in the cat prostate and Schlavonus (1893) and Timofeew (1894) described a very dense nerve plexus in the vas deferens of different mammals The histology of the nerve plexa in question was reexamined by Muller and Dahl (1912), who demonstrated ganglion cells in various parts of the pelvic plexus of man They found no nerve cells within the internal genital organs but observed many nonmyelinated nerve fibres in them. Muller and Dahl concluded that the prostate, the seminal vesicles and the vas deferens were innervated chiefly by nonmyelinated fibres presumably originating from the pelvic nerve cells

Learmonth (1931) concluded that the inferior mesenteric ganglion was absent or vestigial in man but pointed out the hypogastric ganglia at each side of the rectum which receive preganglionic fibres from both the lumbar sympathetic outflow and the sacral parasympathetic outflow He observed that stimulation of the sympathetic fibres produced contraction of the smooth muscles of the prostate, the seminal vesicles and the ejaculatory ducts Learmonth further suggested that in man the preganglionic fibres to these organs originated at the level of the first lumbar ganglion Recent studies have however, indicated that the preganglionic fibres may originate at levels from the last thoracic to the third lumbar ganglion (Whitelaw and Smithwick 1951)

Resection of the lumbar ganglia, the inferior mesenteric ganglion and the hypogastric nerves of man and other mammals has been found to abolish emission by many investigators (Remy 1886, Bacq 1931, Bacq and Brouha 1932, Learmonth 1931 Simeone 1933, van Duzen, Slaughter and White

1947, Whitelaw and Smithwick 1951) Bacq (1931) further found that in rodents, resection of the lumbar sympathetic chain caused temporary sterility of the males while excision of the inferior mesenteric ganglion and the hypogastric nerves resulted in permanent sterility Because regeneration after preganglionic denervation is rather rapid and complete in contrast to regeneration after postganglionic denervation (Langley 1897), Bacq assumed that resection of the inferior mesenteric ganglion involved a break of the postsynaptic links to the male genital organs while excision of the sympathetic chain merely implyed a break of the presynaptic links

Collip (1929) reported on a pressor substance similar to adrenaline, in the "prostatic gland of the bull Euler (1934) showed that the prostate seminal vesicle and the ampulla of the ductus deferens of various species contained relatively large amounts of adrenaline like material Euler further described chromaffin cells in the prostate of the cat and postulated that such cells were the source of the adrenaline like material found in the accessory male glands Later Euler (1961) found noradrenaline in large amounts in the seminal vesicle of the steer

Electrophysiological studies have confirmed the statement of Langley and Anderson (1894) that most fibres in the hypogastric trunk are nonmyelinated Adrian Bronk and Phillips (1932) using cats and rabbits found discharges in slow-conducting fibres of the C-group in the hypogastric nerve but no activity of fibres of the B-group Lloyd (1937) investigated the pre and postganglionic pathways through the inferior mesenteric ganglion in the cat Only a few B-fibres were found in the hypogastric nerve These fibres were probably going straight through the ganglion The vast majority of fibres in the hypogastric nerve were C-fibres relayed in the inferior mesenteric ganglion Grundfest and Gasser (1938) found that the C-fibres elevation made up 90 % of the spike area in the cat hypogastric nerve Burnstock and Holman (1961) in their fundamental paper on autonomic nerve smooth muscle transmission found the average conduction velocity in the guinea pig hypogastric nerve to be 0 9 m per sec which agrees well with that of sympathetic C-fibres Therefore they presumed that most fibres in the nerve to the vas deferens were postganglionic

From the reviewed literature it is concluded that the vas deferens and the accessory male genital glands receive their motor innervation from the lumbar sympathetic outflow and in the case of primates probably also from the last thoracic root The sympathetic fibres reach the pelvis via the hypogastric nerves The localization of the postganglionic neurons is, however not clarified and although some early investigators have suggested that there are synaptic relays close to the target organs, the general opinion—as judged

from textbooks—has been that the postganglionic fibres emanate from the inferior mesenteric ganglion This view seems mainly to be based on the postganglionic characteristics of the hypogastric nerve In man the hypogastric ganglion has been suggested to be the locus of the postganglionic sympathetic nerve cells Earlier studies have also indicated the presence of relatively large amounts of catecholamines in the accessory male organs, but their nature and localization has not been clarified Further previous investigators have demonstrated rather dense nerve plexa in these organs and also nerve cells in their close vicinity, but the nature of these nervous structures has hitherto been obscure

During the last years the sympathetic innervation of the vas deferens has arsoused new interest This is chiefly due to the frequent use of the isolated guinea pig vas deferens—hypogastric nerve preparation, introduced by Hukovic (1961), as a model system for studies on adrenergic transmission These recent reports will, however, be considered later in the present work

CHAPTER II

ANATOMICAL SURVEY

1 NOMENCLATURE

Throughout this report the conventional nomenclature used by physiologists working in this field has been used This nomenclature does not always agree with that used in anatomical textbooks Here some dissimilarities will be clarified and some common synonyms will be given

The term vas deferens is used instead of ductus deferens

The term seminal vesicle *(vesicula seminalis)* is used instead of vesicular gland *(glandula vesicularis—vesiculosa—)*

The term inferior mesenteric ganglion *(ganglion mesentericum inferius)* is used instead of posterior mesenteric ganglion *(G mesentericum posterius)* and caudal mesenteric ganglion *(G mesentericum caudale)* (Some French speaking authors call this ganglion the hypogastric ganglion)

The term prostatic gland which has been used indiscriminately by several workers in referring to various male sex glands has been avoided and accurate terms have been used instead The same holds for the term "prostatic vesicle

The term pelvic plexus has been restricted to mean the meshwork of nerve fibres innervating the pelvic organs but does not include the main sources of this meshwork namely the hypogastric and the pelvic nerves

There is great disunity concerning the terminology of the lumbar and pelvic autonomic structures in man and the macaque The terminology used by Learmonth (1931) and generally used by surgeons has been adopted in this investigation since it is much more expressive than that commonly used by anatomists Thus rather vacant terms such as inferior mesenteric plexus superior hypogastric plexus lower aortic plexus inferior hypogastric plexus etc have been replaced by presacral nerve hypogastric nerve and hypogastric ganglion Concerning the last term anatomists often include the mass of nerve cells at the lateral aspect of the rectum where the hypogastric nerve and the sacral parasympathetic nerves meet in the more diffuse concepts of inferior hypogastric plexus and pelvic plexus In the crab-eating macaques used in this investigation there was a discrete ganglion (demonstrable both macroscopically and microscopically) delineated from the nerves entering and leaving it Therefore the term hypogastric ganglion used by Learmonth in man seems to be adequate and the ganglion should be separated from other pelvic autonomic structures

2 VAS DEFERENS AND ACCESSORY MALE GENITAL GLANDS

The structure and gross appearance of the vas deferens show rather few variations among mammals The variations seen are chiefly in thickness of the muscularis and length of the organ, which depends on the localization of the testis Another factor of variation is the presence of a glandular ampulla or not

The accessory genital glands, however, show great variation among mammals From an embryonic point of view they can be grouped into those which, like the vas deferens, arise from the Wolffian duct, i e the ampullary glands, generally located in the ampulla of ductus deferens and the seminal vesicles, and those which derive from the urogenital sinus or urethra namely the prostate and the bulbo-urethral (Cowpers) glands The accessory male genital glands also include the glands of Littre along the urethra and the preputial glands, which, however, will not be considered in this report

The structure of the seminal vesicle varies from merely a ' blind sac", as seen in the rabbit and the guinea pig, to a compact tubulo-alveolar gland, as seen in the boar In man and other primates, as well as ruminants, intermediate forms are present, with a convoluted sac possessing more or less prominent diverticuli Carnivores lack seminal vesicles

Of the accessory genital glands the prostate or the complex of prostate glands is the one subject to widest variation All types of prostates are commonly composed of tubulo-alveolar glands, but the structure may vary from a discrete "body , with more or less distinct lobes, to a disseminated gland, with the parenchyma spread along the urethra, as seen in ruminants Furthermore, many mammals possess a special gland, the coagulating gland, belonging to the prostate complex and appearing as a discrete gland

The bulbo-urethral glands are covered with striated muscle, in contrast to the other glands, which are compressed by smooth muscle, and show, therefore, a fundamental dissimilarity from them

Special description of vas deferens and accessory male genital glands of examined species

Hedgehog (Fig 1)

The hedgehog has a short vas deferens (V D), since its testes leave the abdominal cavity only during estrus According to Rauther (1904) it has no seminal vesicles but three discrete pairs of prostate glands The first prostate I (P I) (by some authors

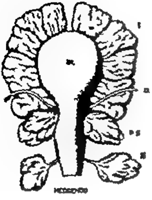


Fig 1

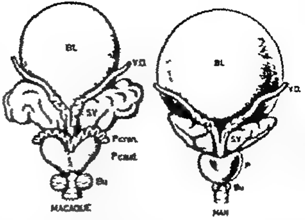


Fig 2

Fig 1 Accessory male genital glands of the hedgehog Ventral view Bl = bladder V.D = vas deferens P I = Prostate I (Seminal vesicle) P II = prostate II P III = prostate III For further information see text[1] *Fig 2* Accessory male genital glands of the macaque compared with those of man. Dorsal view Bl = bladder V.D = vas deferens S.V = seminal vesicle P.cran = cranial prostate P.caud = caudal prostate Bu = bulbourethral gland For further information see text[1]

called seminal vesicle) is a large tubuloalveolar gland surrounding the neck of the bladder Each gland has several lobes and each lobe an excretory duct opening in the urethra The gland has no distinct surrounding muscular coat and the smooth muscular layer of the alveoli is thin especially during estrus when the gland is filled with a rather viscous secretion The other two pairs of prostates prostate II (P II) and prostate III (P III) are similar in gross structure They have only one lobe and the lobe is surrounded by smooth muscle forming a rather thin capsula The prostate I is located below the prostate I but inside the abdominal cavity The prostate III is situated outside the abdomen. The bulbourethral glands are enclosed by the striated muscle of the urethra

Macaque (*Fig* 2)

The vas deferens (V D) of the macaque is rather long since the testes of the adult macaque are located in a scrotum There is no distinct ampulla in its proximal part The seminal vesicles (S V) are rather dense lobulated glands The prostate consists of two distinct lobes The cranial lobe (P cran.) is a lobulated gland grossly resembling the seminal vesicles Since its secretion coagulates the secretion of the seminal vesicles it might be called coagulating gland (v Wagenen 1936) The caudal lobe (P caud) is compact resembling the human prostate but lies entirely on the dorsal aspect of the urethra On either side of the urethra is an oval Cowper s gland (Bu)

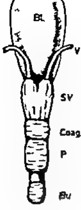


Fig 3

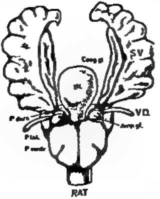


Fig 4

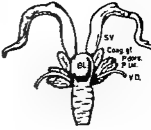


Fig 5

Fig 3 Accessory male genital glands of the rabbit. Dorsal view Bl = bladder V D = vas deferens S V = seminal vesicle Coag gl = coagulating gland P = prostate Bu = bulbo-urethral gland For further information see text[1] *Fig 4 Accessory male genital glands* of the rat Ventral view Bl = bladder V D = vas deferens S V = seminal vesicle Amp gl = ampullary gland Coag gl = coagulating gland P ventr = ventral prostate P lat = lateral lobe of prostate P dors = dorsal lobe of prostate For further information see text! *Fig 5* Accessory male genital glands of the guinea pig Ventral view Bl – bladder V D = vas deferens S V = seminal vesicle P dors = dorsal prostate P lat = lateral prostate Coag gl = coagulating gland For further information see text[1]

Rabbit (Fig 3)

The rabbit vas deferens (V D) resembles that of the macaque It has a slight am pullary enlargement The seminal vesicle (S V) is a large unpaired sacculous gland with a thick smooth muscular wall On the dorsal aspect of the seminal vesicle and in its dorsal wall lies a lobulated coagulating gland (Coag gl) Continous to it the lobulated prostate (P) is situated Below this complex of glands the bulbo-urethral gland (Bu) is located (In front of the seminal vesicles the small paraprostates are situated which microscopically are similar to the Cowpers glands In the examined specimens they were diminute if present at all)

Rat (Fig 4)

The rat has a rather short vas deferens (V D) although it has descended testes but wide inguinal canals Around its proximal part is a small discrete ampullary gland (Amp gl.) almost encircling the ductus deferens The seminal vesicles (S V) are large extended with secretion They are hook-shaped and somewhat convoluted They possess rather thin walls of smooth muscles Along the concavity of each vesicle is a small elongated coagulating gland (Coag gl) The prostate gland has two ventral lobes (P ventr) at the neck of the bladder and two dorsolateral groups of prostatic

acini (P lat and P dors) The prostate of the rat is largely composed of secretory cells and has rather few smooth muscle cells in the walls of the acini especially the ventral ones The small bulbo urethral glands lie between the bulbo and ischio-cavernosus muscles at the base of the penis

Mouse

The accessory glands of the mouse resemble in general those of the rat

Guinea pig (Fig 5)

The guinea pig has its testes in the abdominal cavity and a short muscular vas deferens (V D) without any ampulla The seminal vesicles (S V) are large sacs filled with viscous secretion They possess rather thick muscular walls At the base of the seminal vesicles is the prostate complex consisting of three pairs of rather small tubulo alveolar glands a dorsal prostate (P dors) a lateral prostate (P lat) and an inner coagulating gland (Coag gl) in structure coarser than the other prostates The Cowper s glands lie as they do in the rat

Dog (Fig 6)

The dog has a long vas deferens (V D) since its testes lie in a scrotum It has a prominent circular muscle layer but thin longitudinal layers The vas deferens has a

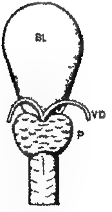


Fig 6

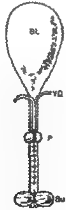


Fig 7

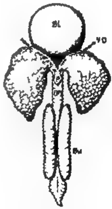


Fig 8

Fig 6 Accessory male genital gland of the dog Dorsal view Bl=bladder V D =vas deferens P =prostate For further information see text[1] *Fig 7* Accessory male genital glands of the cat Dorsal view Bl=bladder V D =vas deferens P =prostate Bu=bulbo urethral gland For further information see text[1] *Fig 8* Accessory male genital glands of the boar Dorsal view Bl=bladder V D =vas deferens S V =seminal vesicle P= prostate body (P)=disseminated prostate Bu=bulbo-urethral gland For further information see text[1]

narrow ampulla The prostate (P) is relatively large and has two lateral lobes surrounded by a muscular capsule which is thicker in the dorsal parts The dorsal parts also have more prominent muscular septa than the ventral and lateral parts have Old dogs generally show a marked hyperplasia of the prostate The dog lacks seminal vesicles and bulbo-urethral glands

Fox

The genital apparatus of the fox is similar to that of the dog but the vas deferens is more slender as it is in the cat

Cat (Fig 7)

The cat vas deferens (V D) is a very long and slender tube Its connective tissue sheeth is rather loose and there is no marked border between it and the tissue sheaths of the other structures in the spermatic cord There is no distinct ampulla The prostate (P) is a small bilobed muscular organ lying on the dorsal wall of the urethra Like all carnivora the cat lacks seminal vesicles The cat has a pair of bulbo urethral glands (Bu) covered by fibres from the bulbo-cavernosus muscle

Boar (Fig 8)

The boar has a long vas deferens (V D) which forms no distinct ampulla The seminal vesicles (S V) are exceedingly large glands of a branched tubular structure They have no distinct muscular capsule and rather few muscle cells in the stroma The prostate (P) consists of a rather small body at the dorsal aspect of the urethra and a disseminated part surrounding the pelvic part of the urethra and covered by the urethral muscle The bulbo-urethral glands (Bu) are very large cylindrical glands partially covered with the striated bulbo-glandularis muscle

Fig 9

Fig 10

Fig 9 Accessory male genital glands of the bull Dorsal view Bl = bladder V D = vas deferens A = ampulla of vas deferens S V = seminal vesicle P body = prostate body P diss = disseminated prostate Bu = bulbo-urethral gland For further information see text!

Fig 10 Accessory male genital glands of the ram Dorsal view Bl = bladder V D = vas deferens A = ampulla of vas deferens S V = seminal vesicle P diss = region of urethra containing the disseminated prostate glands Bu = bulbo-urethral gland For further information see text!

Bull (Fig 9)

The vas deferens (V D) of the bull is rather small in caliber It forms a prominent ampullary enlargement (A) with a plicated mucosa and a thick muscular wall The seminal vesicles (S V) are compact lobulated glands The bull has a small prostate body (P body) but a rather long disseminated part (P diss) The two bulbourethral glands are rather small

Ram (Fig 10)

The vas deferens (V D) and the genital glands of the ram resemble in general those of the bull Tre prostate (P diss) is entirely disseminated Like the disseminated glands of the boar and the bull there is however rather large amounts of unstriped muscle in the interlobular tissue The Cowper s glands (Bu) are relatively large, but the seminal vesicles (S V) are relatively small compared with those of the bull

As seen from the special description there is a great variation in both the number of accessory genital glands and in the structure of homologous organs of different species

For further discussion in this investigation the author found it suitable to group the glands grossly after the development of their muscular compartments Most accessory male reproductive glands have well-developed smooth muscle layers surrounding the secretory mucosa and thus resemble the structure of the vas deferens Prominent examples of this type of gland are the seminal vesicles of the rabbit and the ruminants This type of structure is also the dominating one among the glands of the various prostates although in these glands the secretory compartments are relatively larger than in the seminal vesicles However, among the accessory male glands investigated there are some which show a rather weak development of the smooth muscle compared to the secretory compartments These glands are the prostate I of the hedgehog and the seminal vesicle of the boar and parts especially the ventral ones of the prostates of the rat and the dog Concerning the rat it should be noted that the smooth muscle layers of the accessory male glands are relatively weaker than those of the homologuos glands in the rabbit and the guinea pig The prostates II and III of the hedgehog also have rather thin smooth muscle layers during estrus

Finally as mentioned above, in the bulbo-urethral glands the smooth muscle fibres are to a great extent replaced by striated muscle fibres

For further information on this subject the reader is referred to the extensive monograph by Disselhorst (1904) Recent accounts of parts of this subject have been given by Price and Williams-Ashman (1961) and Price (1963)

3 INNERVATION OF VAS DEFERENS AND ACCESSORY MALE GENITAL GLANDS

The pelvic organs receive nerve fibres from the sympathetic and the parasympathetic nervous systems Sympathetic fibres reach the pelvic organs via the hypogastric nerves, which emanate from the inferior mesenteric ganglion (or ganglia) In addition to this come some fibres emanating from the sympathetic chain The parasympathetic innervation derives from the sacral outflow With the exception of the macaque and man the detailed innervation pattern is very similar in the examined species (Fig 11 A)

The inferior mesenteric ganglion is located on or beneath the base of the inferior mesenteric artery It may form a compact mass of nerve cells or may be split into two, four or more small ganglia, as is the rule in the cat and the rabbit The ganglion receives fibres from the lumbar sympathetic ganglia and some fibres from the upper abdominal ganglia From the ganglion two hypogastric nerves, one left and one right emerge The hypogastric nerves may diverge during their course into secondary branches This is regularly seen in ruminants and sometimes in the guinea pig but is rare in other species In the rabbit the two hypogastric nerves are generally combined in their proximal parts into one nerve When the hypogastric nerves reach the pelvic organs they split off forming a nervous plexus (pelvic plexus) This plexus is situated in the connective tissue close to the internal genital organs the bladder and the rectum and supplies these organs

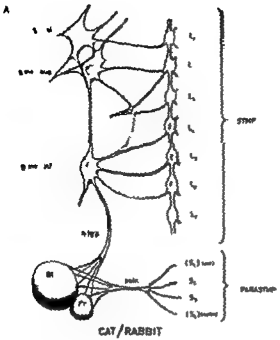
Fibres from the sympathetic chain chiefly follow the arteries to the pelvic organs or join the parasympathetic fibres

The sacral parasympathetic fibres generally combine forming the two pelvic nerves (a left and a right one), which intermingle with the hypogastric nerve fibres, when they reach the pelvic organs In the *guinea pig* the pelvic nerve runs to the bladder and seems not to take part in the nervous plexus innervating the internal genital organs

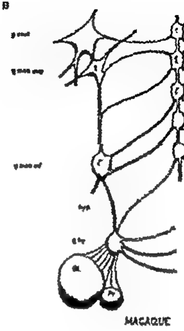
The *macaque* (Fig 11 B) diverges from this pattern, since in this animal the sacral nerves join the hypogastric nerves and form two hypogastric ganglia from which nerve fibres emerge and innervate the pelvic organs Thus the innervation pattern of this animal represents an intermediate form between lower mammals and man (see below), since the macaque has a well developed inferior mesenteric ganglion two discrete hypogastric nerves and two distinct hypogastric ganglia

In man (Fig 11 C) the inferior mesenteric ganglion is absent or vestigial The hypogastric nerves are branches of the presacral nerve which is composed of fibres from

A



B



C

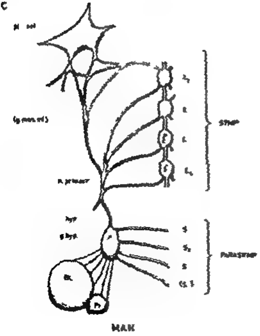


Fig 11 Schematic drawings illustrating the main sympathetic and the parasympathetic innervation of the male urogenital system in different species

A Pattern in lower mammals as the cat and the rabbit.

B Pattern in the macaque

C. Pattern in man

Dotted lines indicate connections which may be weak or absent. Bl = bladder Pr = prostate vas deferens and seminal vesicle g coel = coeliac ganglia pl.coel = coeliac and allied ganglia g mes sup = superior mesenteric ganglion g mes inf = inferior mesenteric ganglion(ia) n hyp = hypogastric nerve n.presacr = presacral nerve g hyp = hypogastric ganglion n pelv = pelvic nerve

the intermesenteric and inferior mesenteric plexa (and ganglion if present), the middle root, and fibres from the lumbar ganglia of the sympathetic chain, the lateral roots. About 60 per cent of the fibres in the presacral nerve are medullated (Learmonth 1931) most of them comming from the lateral roots Each hypogastric nerve ends in a hypogastric ganglion situated on the lateral aspect of the rectum The sacral parasympathetic rami also end in this ganglion from which the pelvic organs are innervated.

Accounts of this subject are to be found in the papers by Langley and Anderson (1896), Learmonth (1931), Trumble (1934) and in the monograph by Mitchell (1953)

CHAPTER III

MATERIAL AND METHODS

Material

The present investigation deals with the common experimental animals (mouse, rat, guinea pig, rabbit, cat and dog) and domestic animals (boar bull and ram) In addition, human vasa deferentia were added as well as material from another primate the macaque, having an innervation pattern resembling man The hedgehog was investigated as being a rather primitive placental mammal Since the dog proved to diverge from the other mammals, its relative, the fox was also investigated All animals were not subjected to all types of investigation thus experiments with isolated innervated organs were performed on the guinea pig for anatomical reasons only and total lumbosacral sympathectomy was performed on the cat only A schematic disposition of the material and the investigations are given in Table 1

The following species were used in the investigation

ORDER	EXAMINED SPECIES
Insectivora	Hedgehog *(Erinaceus Europeus)*
Primates	Crab-eating macaque *(Macaca irus (cynomolgus))*
	Man *(Homo sapiens)*
Lagomorfa	Rabbit *(Oryctolagus cuniculus dom)*
Rodentia	Mouse *(Mus musculus)*
	Rat *(Rattus norvegicus)*
	Guinea pig *(Cavia cobaya (porcellus))*
Carnivora	Dog *(Canis familiaris)*
	Arctic fox *(Alopex (Canis) lagopus)*
	Cat *(Felis catus)*
Artiodactyla	Boar *(Sus scrofa dom)*
	Bull *(Bos taurus)*
	Ram *(Ovis aries)*

With the exception of the two macacques used in the histo-chemical study all animals used were adult The *hedgehogs* were taken in the end of June

which is supposed to be in the end of their estrus, yet their glands were full of secretion The *human* vasa deferentia were taken from old men operated for prostatic hypertrophy or cancer The *rabbits* were of the small chinchilla race or crossings between this race and other pigmented races The *rats* were of the Sprague Dawley strain The *mice* were of the N M R I -strain The *dogs* were hunters (Pointers, Beaglers, Vorsteh dogs, Harriers and Retrievers) or crossings between Harriers and other big dog races The *foxes* were

Table I Survey of the investigation and the material

Investigation	*Species*	*Number of animals*
Isolated vas deferens-hypogastric nerve preparation		
Intact animals	Guinea pig	32
Decentralized animals	Guinea pig	10
Estimation of catecholamines		
Intact animals	Hedgehog	3
	Macaque	4
	Man	5
	Rabbit	8
	Mouse	15 (3 × 5)
	Rat	4
	Guinea pig	10
	Dog	6
	Fox	3
	Cat	8
	Boar	3
	Bull	3
	Ram	3
Hypogastr den	Macaque	1
	Rabbit	4
	Rat	5
	Guinea pig	10
	Dog	5
	Cat	6
	Ram	2
Pelv den.	Dog	2
Hypogastr + lumbosacral sympathectomy	Cat	6

Table I Continued

Fluorescence microscopy		
Intact animals	Macaque	2
	Rabbit	3
	Rat	3
	Guinea pig	5
	Dog	1
	Cat	2
Unilat hyp den	Rabbit	2
	Rat	4
	Guinea pig	9
	Dog	1
	Cat	1
Bilat hyp den	Rabbit	3
	Rat	3
	Guinea pig	8
	Dog	1
	Cat	2
Hyp-den + lumbosacral sympathectomy	Cat	2
Nialamide	Rabbit	2

domestic blue foxes Organs from young *boars* of the Yorkshire stock and young *bulls* of the Swedish black mottled lowland cattle were used Young *rams* of the "Gotland race were investigated

Operative procedures

A Hypogastric denervation

Rabbits were pretreated with atropine (2 mg/kg) and subjected to hypogastric denervation under ether anesthesia Through a midline incision the hypogastric nerves and parts of the inferior mesenteric ganglion were removed from the level of the inferior mesenteric ganglion down to the point where the branches run close to the rectum

Rats and *guinea pigs* were submitted to hypogastric denervation under nembutal anesthesia supplemented when necessary with ether The abdomen was opened with a midline incision and the hypogastric nerves were removed from their origin at the inferior mesenteric ganglion to a point close

to the seminal vesicles where they join the arterial supply of the internal accessory male genital organs After thus removing about 5 cm of the nerves in the guinea pig and about 2 cm in the rat the abdomen was closed

Dogs were operated under nembutal anesthesia About 6 cm of the hypogastric nerves were removed through an abdominal incision on the left side After the operation the abdomen was carefully closed layer by layer

Cats were anesthetized with nembutal The abdomen was opened in the midline and the hypogastric nerves and the inferior mesenteric ganglia encircling the inferior mesenteric artery were identified The ganglia and about 5 cm of the hypogastric nerves were removed and the abdomen was closed

Rams which had been starved for 2 days were pretreated with atropine (1 mg/kg) 1 h before operation They were given nembutal i v to a rather light anesthesia Full anesthesia was then obtained by ether through a tube inserted in the trachea An incision was made in the left side of the abdomen below the ribbons A small incision was then made in the *rumen* and another in the *omasum* Through these incisions most of the content of the stomach was removed, and the incisions and the abdomen closed Then the abdomen was opened again by a new incision on the right side of the penis, the inferior mesenteric ganglion was identified and removed together with about 5 cm of the hypogastric nerves, after which the abdomen was closed

Only one succesful operation was performed on *macaque* It was made in a similar way as the operations on cats The monkey had to be killed 2 days after operation

In most animals both hypogastric nerves were removed but in some of the animals used for the histochemical investigation only one nerve was removed The animals were sacrificed 6-29 days after the operation The accessory male genital glands of rat, guinea pig and rabbit were then extended and filled with secretions and the vas deferens was filled with spermatozoa

B Pelvic denervation

In two *dogs* subjected to hypogastric denervation the pelvic nerves were also cut The abdominal incision was enlarged, the pelvic nerves were identified and removed from their origin to the place where they join the pelvic plexus The animals were killed 6 days after operation

C Lumbo-sacral sympathectomy

In 8 *cats* submitted to hypogastric denervation lumbo-sacral sympathectomy was performed After abdominal incision the dorsal peritoneum was in

cised and the sympathetic chain removed. In one cat one small ganglion and in another two small ganglia distal to the promontorium escaped removal. Since the most distal ganglia are supposed merely to supply the legs these two cats were used in the material. The cats were killed 6 days post operatively.

D. Decentralization of the inferior mesenteric ganglion

Ten *Guinea pigs* used for the isolated nerve—vas deferens preparation were submitted to decentralization of the inferior mesenteric ganglion. The abdomen was opened by a midline incision and the inferior mesenteric ganglion was identified at its place below the inferior mesenteric artery. The ganglion was gently elevated and its preganglionic roots were cut. Through this procedure all visible roots could be cut but some fibers running too close to the arteries might have escaped detection. The 10 guinea pigs used in the investigation showed no macroscopic granuloma in the operation field when used for organ bath experiments 5-10 days after operation.

The operations were not performed under aseptic conditions but the animals, with the exception of the guinea pigs and the rabbits were treated with 10 000—25 000 units of penicillin per kg daily for 4 days after denervation.

All operated animals, as well as controls, were killed by bleeding under ether or nembutal anesthesia. The guinea pigs used for the isolated vas deferens preparation were, however, killed by a blow on the head.

Isolated vas deferens preparation

The vas deferens with the hypogastric nerve was prepared according to the method described by Huković (1961). The organ was placed in a 50 or 40 ml bath containing Tyrode solution at 37° C and aerated with 6.5 % CO_2 in O_2. The contractions were recorded on a smoked drum with a frontal writing lever (magnification 1:16). The hypogastric nerve was placed on stimulating electrodes (2 mm between the two platinum electrodes). In one series of experiments the nerve and electrodes were immersed in liquid paraffin. In another the nerve and the electrodes were kept in the solution of the bath. The nerve was stimulated every one or two min for 5 to 10 sec with supramaximal voltage (10-15 V if the nerve was in paraffine oil, 30-100 V if the nerve was in the Tyrode solution) and a duration of 2 msec, the frequency being in the range 5-20 per sec. In most experiments the electrodes were placed about 4 cm apart from the organ. The average weight of the guinea pigs was 500 g, and each preparation could be used for at least 5-6 h. Each type of experiment was undertaken on *vasa deferentia* from 5-15 different animals. The solutions used were acetylcholine chloride 10 and 1

mg/ml atropine sulphate 0 1 mg/ml azamethonium hydrochloride (Pendomid) 1 mg/ml hexamethonium hydrobromide 1 mg/ml lobeline hydrochloride 1 and 0 1 mg/ml nicotine bitartrate 1 and 0 1 mg/ml noradrenaline hydrochloride 10 and 1 mg/ml and tetraethylammonium hydrobromide 10 and 1 mg/ml All substances were in aqueous solution.

The Tyrode solution had the following composition 0 8 per cent NaCl 0 02 per cent KCl 0 02 per cent $CaCl_2$, 0 01 per cent MgCl 0 1 per cent NaHCO 0 005 per cent NaH_2PO_4 H O and 0 1 per cent glucose

Estimation of catecholamines

The organs were taken out immediately after death and cleaned Their contents of secretions and spermatozoa were squeezed out as much as possible and the organs were weighed If the weight exceeded 2 g only about 1 g was used for analysis In this case pieces were taken from at least 4 different sites of the organ Each determination on mice was made on pooled organs from 5 mice Organs from bulls and boars were frozen immediately after slaughter and extracted as soon as possible generally within 4 h The pieces from human vasa deferentia were extracted within 5 h after removal, but they were not frozen

After weighing the organs were homogenized with an Ultra Turrax apparatus in 20 ml of 10 per cent trichloroacetic acid After 30 min extraction the organ extracts were filtered and the catecholamines were adsorbed on columns of alumina eluted and estimated according to the method of Euler and Lishajko (1961) Recovery experiments with a pooled extract of seminal vesicles from the guinea pig yielded 88 95 per cent of added amounts of noradrenaline (1—2 μg) The mean of 6 expts being 89 6 per cent The extracted catecholamines were identified in an Aminco Bowman spectrophotofluorimeter

The accuracy and specificity of this method has been discussed in detail by Euler and Lishajko (1961) and Gunne (1963)

Fluorescence microscopy

This part of the investigation consists of

22 *guinea pigs* weighing about 500 g (a few young ones weighing about 200 g) Of these the hypogastric nerves were removed bilaterally in 8 and on only the left side on 9 Five animals served as untreated controls

10 *rats* of about 250 g weight Three were submitted to bilateral section of the hypogastric nerves, 4 to unilateral on the left side and 3 served as controls

10 *rabbits* weighing 2 3 kg Three of them were subjected to bilateral hypogastric denervation in 2 others only the left branch was cut. Three animals were left as untreated controls Finally 2 animals received a monoamine oxidase inhibitor—nialamide—(300 mg/kg i p) followed after 2 h by 1 DOPA (100 mg/kg i v) The animals were killed 20 min after the last injection

7 *cats* weighing more than 3 kg Two animals served as controls In one animal only the right hypogastric nerve was removed One cat was submitted to bilateral hypogastric denervation In 2 cats bilateral hypogastric denervation and total lumbosacral sympa

thectomy was performed. Finally in one animal in addition to bilateral hypogastric denervation an attempt was made to remove the connective tissue between the vas deferens and the prostate on the right side (in this tissue area where the hypogastric nerve is branching adrenergic nerve cell bodies are present *cf.* Chapter VI).

3 *dogs* weighing 23-30 kg. In one of them both hypogastric nerves were excised, in another only the left one. The remaining dog served as control.

2 intact young *macaques* weighing about 1 kg. They were not fully sexually mature since their testis still remained in the inguinal canal.

After death of the animals the following tissues were immediately dissected out: the vas deferens, the internal accessory male genital glands and the distal parts of the hypogastric nerves, where they divide to form the nervous plexuses supplying the internal male genital organs. The tissue pieces were frozen in propan cooled with liquid nitrogen and after freezedrying treated in formaldehyde gas, some for 1 h and others for 3 h and finally infiltrated with paraffin *in vacuo*. Tissue sections (6 μ) were mounted for fluorescence microscopy in Merck's Entellan (slightly diluted with xylene) or in non-fluorescent liquid paraffin. The detailed procedure is described elsewhere (Falck 1962, Falck and Owman 1965).

The monoamines condense at their cellular storage sites with formaldehyde into highly fluorescent products. In case of primary catecholamines and 5-hydroxytryptamine an intense fluorescence is readily obtained within 1 h exposure to formaldehyde gas, whereas adrenaline needs a prolonged treatment to develop a maximal fluorescence. The fluorophores are insoluble in paraffin but that of adrenaline is, in contrast to the others, soluble in organic solvents as xylene. With the filter combinations used, the fluorophores of the catecholamines emit a green light and that of 5-hydroxytryptamine emits a yellow light. However, certain cells, containing very high amounts of catecholamines display with increasing time of exposure a fluorescence in the yellow to orange range (*cf.* Enemar, Falck and Håkansson 1965). Further the fluorophore of 5-hydroxytryptamine shows a marked UV lability in comparison to the fluorophores of the catecholamines. Thus, 5-hydroxytryptamine and adrenaline can be differentiated from the primary catecholamines (*cf.* Falck and Owman 1965).

The chemical background of the reaction and the specificity of the method has been discussed at length elsewhere (Falck *et al.* 1962, Falck 1962, Corrodi and Hillarp 1963, 1964, Falck 1964, Norberg and Hamberger 1964, Falck and Owman 1965). From these data it appears that the method has a high specificity as well as a high sensitivity for certain biogenic monoamines, enabling, for example, the demonstration of intraneuronally located noradrenaline (Falck and Torp 1962).

Further, the vas deferens from dog was silver impregnated according to Bodian (1936) for the visualization of nerve fibres. Freeze-dried tissue pieces fixed in formaldehyde gas from paraformaldehyde were embedded in paraffin, sectioned transversely at 6 μ, and then silver impregnated by double impregnation.

Calculation of values and statistical treatment

The catecholamine values in this report are expressed as hydrochlorides. The tissue contents of catecholamines were calculated as the amount per gram of wet tissue weight.

Statistical treatment was performed on experimental groups exceeding 3 observations Mean and standard error were computed according to ordinary formulae (Fisher 1936) Significance of means and differences were tested by the Student t test (Fisher 1936), on the assumption that the mean difference was not significant

CHAPTER IV

EFFECT OF GANGLIONIC BLOCKING AGENTS ON THE MOTOR RESPONSE OF THE ISOLATED GUINEA PIG VAS DEFERENS TO HYPOGASTRIC NERVE STIMULATION

The experiments described in this chapter were originally initiated by findings apparently supporting the theory of Burn and Rand (Burn 1960 Burn and Rand 1960) that a cholinergic mechanism caused the peripheral release of the adrenergic transmitter Thus Boyd Chang and Rand (1960) found that cholinesterase inhibitors increased the motor response of the vas deferens to hypogastric nerve stimulation and Rand and Chang 1960 found that hemicholinium inhibited this response On the other hand Burnstock and Holman (1960) and Rand (1961) saw no effect of hexamethonium on this response Taken together, these findings would favour the idea that the adrenergic transmitter was released by a *nonganglionic* cholinergic mechanism The present author found it however necessary to investigate further the effect of ganglionic blocking agents on the response to nerve stimulation of the guinea pig vas deferens

RESULTS

Hexamethonium in doses of 1 20μg/ml inhibited the response of the isolated guinea pig vas deferens to hypogastric nerve stimulation Small doses 1 5

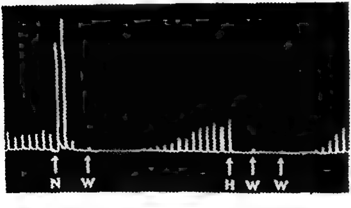


Fig 12 Contractions of the isolated guinea pig vas deferens in response to nerve stimulation of supramaximal voltage (15 V 2 msec 5 pulses per sec for 5 sec at 2 min intervals) bath volume 50 ml N = 200 μg nicotine H = 250 μg hexamethonium at W washing

μg/ml generally diminished the contractions while larger doses 3 20 μg/ml *abolished the response to nerve stimulation completely* (Fig 12) The onset of the inhibition was dependent on the dose of hexamethonium, thus when larger doses were used total inhibition appeared immediately and when smaller doses were used the inhibition was gradual After washing the bath the contractions reappeared in 15 30 min and regained their initial heights in 30 40 min, depending on the dose When doses of 10 20 μg/ml had been added to the bath the contractions often never reached their initial size The hexamethonium block could partly be overcome if the stimulation frequency was increased from the initial 5 20 per sec to 30 60 per sec (Fig 13), If more hexamethonium was then added to the bath, the contractions again diminished When a very high stimulation frequency (60 100 per sec) was used, the block caused by hexamethonium was incomplete, and partial recovery soon appeared, even if there was a high concentration of hexamethonium in the bath In this case addition of a small dose of atropine (0 1 1 μg/ml) often abolished the contractions (Fig 14)

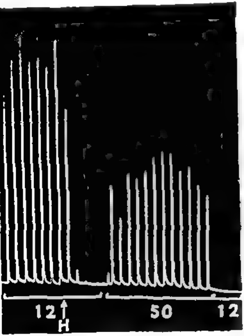


Fig 13 Contractions of the isolated guinea pig vas deferens in response to nerve stimulation of supramaximal voltage (15 V,2 msec for 5 sec at 2 min intervals) bath volume 50 ml Stimulation frequency indicated by number under contractions at *H* addition of 500 μg hexamethonium

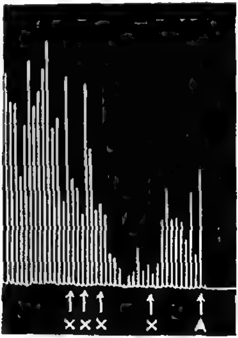


Fig 14 Contractions of the isolated guinea pig vas deferens in response to nerve stimulation of supramaximal voltage (15 V 2 msec 100 pulses per sec for 0 5 sec at 0 5 min intervals) bath volume 50 ml X = 250 μg hexamethonium A = 5 μg atropine

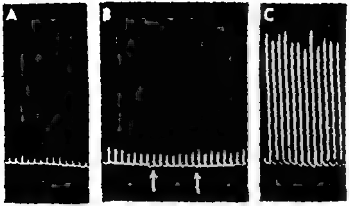


Fig 15 Contractions of the isolated guinea pig vas deferens in response to nerve stimulation of supramaximal voltage (50 V 2 msec for 5 sec at 1 min intervals) bath volume 40 ml *Decentralized preparation*

A Electrodes about 2 5 cm from vas deferens Stimulation frequency 50 pulses per sec

B Electrodes about 1 cm from vas deferens Stimulation frequency 10 pulses per sec At arrows 500 μg hexamethonium

C Electrodes about 0 5 cm from vas deferens Stimulation frequency 10 pulses per sec

If the stimulating electrodes were placed very close to the vas deferens —about 0 5 cm from the organ—hexamethonium caused almost no or only a slight reduction of the contractions

Tetraethylammonium exerted the same effect as hexamethonium but had a weaker action To abolish completely the response to nerve stimulation the concentration in the bath had to exceed 15 μg/ml The time of recovery after washing was shorter than with hexamethonium

Azamethonium (Pendiomid) had the same effect as hexamethonium and was active in the same concentrations (5 20 μg/ml) The effect of azamethonium did, however, never appeare so rapidly as that of hexamethonium and when small doses were used contractions reappeared almost immediately after washing

Nicotine in doses of 0 4 10 μg/ml abolished the response of the isolated guinea pig vas deferens to sympathetic nerve stimulation Generally a period of potentiation preceded the inhibition (Fig 12), but in some preparations no potentiation could be seen Low doses of nicotine (0 2 1 μg/ml) often produced only potentiation, but this was never of great magnitude After washing the bath following a nicotine block the contractions gradually returned If small blocking doses had been used, contractions could

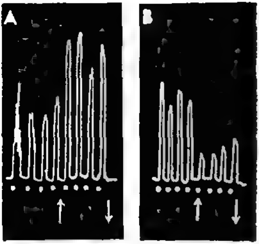


Fig 16 Isolated guinea pig vas deferens bath volume 50 ml

A At dots 500 μg noradrenaline between arrows 1 000 μg hexamethonium in the bath

B At dots 250 μg acetylcholine between arrows 1 000 μg hexamethonium in the bath

be seen after about 15 min and were completely restored after about 30 40 min If large doses of nicotine had been used, complete restoration of the contractions was seldom seen

Lobeline in doses of 5 10 μg/ml obliterated the motor response to hypogastric stimulation The onset of this inhibition was gradual and was not preceded by a period of potentiation The duration of the lobeline block was long, and new contractions were seldom seen until after about an hour Smaller doses of lobeline (1 4 μg/ml) had no effect or exerted a very slight potentiation of the motor response

When the hypogastric nerve from decentralized guinea pigs was stimulated, the vas deferens did not respond until very high frequencies were used (50 100 per sec) These responses were, however, of a very low magnitude (Fig 15A) They were not distinctly reduced by hexamethonium (10 20 μg/ml) (Fig 15B) If the stimulating electrodes now were moved close to the vas deferens—about 0 5 cm apart from the organ— normal responses were obtained on hypogastric nerve stimulation (Fig 15C)

In order to exclude an effect of the ganglionic blocking substance on the transmitter, experiments were performed with isolated innervated *vasa deferentia* stimulated with noradrenaline and acetylcholine Hexamethonium was chosen as being the most active and specific of the ganglionic blocking agents The vas deferens was contracted by noradrenaline and acetylcholine in doses of 5 20 μg/ml Hexamethonium 20 μg/ml increased the response to noradrenaline to about twice the initial size (Fig 16A) but reduced the response to acetylcholine to about half the initial size (Fig 16B)

DISCUSSION

It has been seen in these experiments that the motor response of the vas deferens to hypogastric nerve stimulation is inhibited by several substances known to exert almost specific ganglionic blocking action If the preganglionic rami to the inferior mesenteric ganglion are cut and left to degenerate the response to hypogastric nerve stimulation is almost abolished On the other hand stimulation close to the vas deferens elicits a "normal response, which is not affected by ganglionic blockers

The results indicate that the hypogastric nerve fibres have a synaptic relay close to the vas deferens

Since first reported (Sjostrand 1962 a) these findings have been confirmed by many investigators using different techniques Ferry (1963) using *intracellular microelectrodes showed that the hypogastric fibers evoking* responses from the smooth muscle cells of the guinea pig vas deferens were B fibers Kuriyama (1963), using the same technique, observed that hexamethonium blocked the generation of junction potentials in response to nerve stimulation Bentley and Sabine (1963) and Birmingham and Wilson (1963), working with hypogastric nerve and transluminal stimulation demonstrated that hypogastric nerve stimulation but not transluminal was obliterated by ganglion agents, thus indicating a synaptic relay located close to the vas deferens That hexamethonium blocks the response to hypogastric nerve stimulation was also confirmed by Ohlin and Stromblad (1963) using the same conditions as the present author There is however some disparity concerning the effective concentration of hexamethonium, thus Kuriyama had to use 100 $\mu g/ml$ to obtain a block This might be due to differences in temperature and composition of solutions and could explain why Burnstock and Holman (1960) and Rand (1961) did not observe any effect of hexamethonium

Postganglionic fibers emanating from the inferior mesenteric ganglion do not seem to take part in the innervation of the vas deferens to any appreciable degree, as judged from the results obtained with decentralized preparations On the other hand these results speak in favour of the idea that the hypogastric nerve contains presynaptic fibers passing the inferior mesenteric ganglion and ending on nerve cells in the close vicinity of the vas deferens The slight response of the decentralized preparations observed when very high stimulation frequencies were used could be due to some presynaptic fibers escaping section or perhaps to the presence of a small proportion of postsynaptic fibers having their cell bodies in the inferior mesenteric ganglion or the proximal part of the hypogastric nerve

During recent years pharmacological evidence has accumulated supporting the idea that noradrenaline actually is the transmitter evoking motor responses of the vas deferens upon hypogastric nerve stimulation (Huković 1961, Boyd, Chang and Rand 1960, Bentley 1962, Bentley and Sabine 1963, Birmingham and Wilson 1963 Burnstock and Holman 1964 and others) Thus, obviously the nerve stimulus has to pass a peripheral *ganglionic* cholinergic synapse before it releases the adrenergic transmitter Recent pharmacological and morphological findings (*cf* Chapter VI) seem definitely to exclude any *one of the alternative mechanisms tentatively suggested by the* author, when these results were first presented (Sjostrand 1962 a), i e effect of ganglionic blockers on chromaffin cells or a nonspecific depression of nerve terminals

Acetylcholine acts probably partly on structures which are blocked by hexamethonium, in agreement with previous findings, on the other hand it *seems partly to have a direct action on the muscle cells This could explain* the synergistic action of atropine on hexamethonium when high frequencies of stimulation are used The contractions seen under these conditions might be due to an overflow of acetylcholine when its receptors are blocked Another explanation of this phenomenon is a true synergism of atropine and hexamethonium

Stimulation frequencies in the range 50 100 per sec must, however, be regarded as unphysiological The effect of acetylcholine is, however, confusing, since hexamethonium reduces the acetylcholine induced contractions to about half their initial size, while atropine, 0 1 $\mu g/ml$ (Boyd, Chang and Rand 1960), completely obliterates these contractions Atropine in this concentration exerts almost no effect on the response to nerve stimulation These *facts do not conform with the classical concept of the muscarinic and nico-*tinic actions of acetylcholine Concerning the effect of nicotine, it is also puzzling that nerve transmission failure is obtained with doses smaller than those which produce contraction of the vas deferens (20 $\mu g/ml$) (Boyd, Chang and Rand 1960)

CHAPTER V

CATECHOLAMINES IN VAS DFFERENS AND ACCESSORY MALE GLANDS OF DIFFERENT SPECIES AND THE EFFECT OF SYMPATHETIC DENERVATION ON THEM

The results described in the previous chapter indicated the existence of a peripheral synapse located close to the effector organ and belonging to the adrenergic innervation of the guinea pig vas deferens If this were the case, section and degeneration of the hypogastric nerve branches to this organ would not be accompanied by the reduction in noradrenaline content which is seen in other organs after postsynaptic sympathetic denervation (Euler and Purkhold 1951, Goodall 1951) The noradrenaline would remain in the organs, as is seen after presynaptic denervation (Rehn 1958) The same would probably be the case if the organs were storing catecholamines in chromaffin cells since denervation of the adrenal medulla does not alter its catecholamine content (Euler and Hokfelt 1950 Hokfelt and McLean 1950)

The investigation was also extended to the accessory male genital glands and to other species than the guinea pig for reasons given in the introduction of this report

RESULTS

The noradrenaline content of the internal accessory male genital organs of the examined species are given in Tables II XIII

In Fig 17 the noradrenaline content of the vasa deferentia of different species are compared Material from the cock (White Leghorn strain) and the Greek tortoise (Sjöstrand 1965) is also included in this figure In Fig 18 and Fig 19, respectively, the contents of the prostates and the seminal vesicles are compared

The effect of hypogastric denervation was studied in rabbit, rat, guinea pig cat dog ram and monkey (only one successful operation) The data are given in Tables III, V, VII, VIII, IX, X and XIII, Figs 20, 21 and 22 give a comparison between denervated and nondenervated specimens As seen from the tables there is only a slight or no reduction in the

Table II Noradrenaline (NA) in vas deferens and accessory male glands of the hedgehog

Hedgehog no	Body weight (kg)	Weight of pair of vasa def (g)	NA in vas def (μg/g tissue)	Weight of pair of prost I (g)	NA in prost I (μg/g tissue)	Weight of pair of prost II (g)	NA in prost II (μg/g tissue)	Weight of pair of prost III (g)	NA in prost III (μg/g tissue)
1	0.72	0.03	7.3	2.38	0.94	1.08	0.95	—	—
2	0.85	0.05	9.0	7.88	0.45*	4.26	1.25	2.3	0.88
3	1.10	0.06	9.7	15.91	0.59	5.12	1.25	3.1	0.62
Mean	0.89	0.05	8.7	8.72	0.66	3.49	1.15	2.7	0.75

* Adrenaline 0.13 μg/g tissue

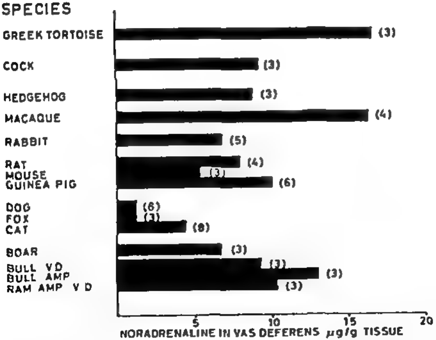


Fig 17 Comparison of the noradrenaline content of the vas deferens of some vertebrates Number of animals within brackets Note the comparatively low content of the dog and fox vas deferens and the comparatively high content of the vasa deferentia of tortoise and macaque

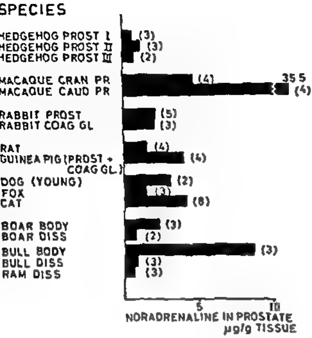


Fig 18 Comparison of the nor adrenaline content of the pro-state(s) of some mammals Number of animals within brackets Note the very high content of the caudal prostate of the macaque and the comparatively low contents of the prostates of the hedgehog and the disseminated prostates of artiodactyles

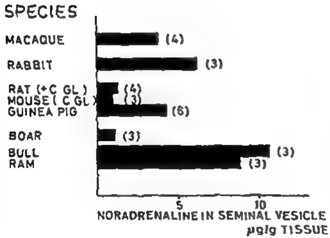


Fig 19 Comparison of the nor adrenaline content of the seminal vesicle of some mammals Note the comparatively low contents of the seminal vesicl s of rat mouse and boar

noradrenaline content following removal of the hypogastric nerves The difference in noradrenaline content between control animals and denervated animals is in no case statistically significant by the Student *t* test The greatest difference is seen in the guinea pig vas deferens where it is 2 0 ± 0 92

Table II *Noradrenaline (NA) in vas deferens and accessory male glands of the hedgehog*

Hedgehog no	Body weight (kg)	Weight of pair of vasa def (g)	NA in vas def (μg/g tissue)	Weight of pair of prost I (g)	NA in prost I (μg/g tissue)	Weight of pair of prost II (g)	NA in prost II (μg/g tissue)	Weight of pair of prost III (g)	NA in prost. III (μg/g tissue)
1	0.72	0.03	7.3	2.38	0.94	1.08	0.95	—	—
2	0.85	0.05	9.0	7.88	0.45*	4.26	1.25	2.3	0.88
3	1.10	0.06	9.7	15.91	0.59	5.12	1.25	3.1	0.62
Mean	0.89	0.05	8.7	8.72	0.66	3.49	1.15	2.7	0.75

* Adrenaline 0.13 μg/g tissue

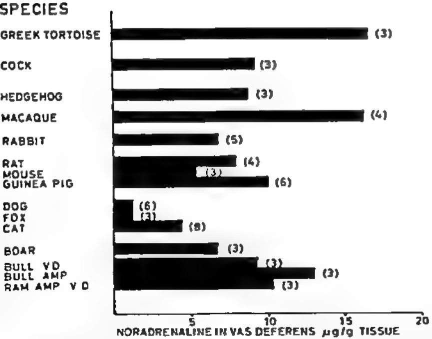


Fig 17 Comparison of the noradrenaline content of the vas deferens of some vertebrates. Number of animals within brackets. Note the comparatively low content of the dog and fox vas deferens and the comparatively high content of the vasa deferentia of tortoise and macaque

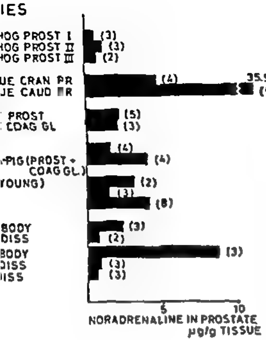


Fig 18 Comparison of the noradrenaline content of the prostate(s) of some mammals. Number of animals within brackets. Note the very high content of the caudal prostate of the macaque and the comparatively low contents of the prostates of the hedgehog and the disseminated prostates of artiodactyles.

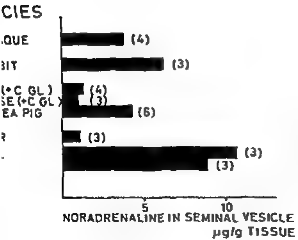


Fig 19 Comparison of the noradrenaline content of the seminal vesicle of some mammals Note the comparatively low contents of the seminal vesicles of rat, mouse and boar

oradrenaline content following removal of the hypogastric nerves The dif rence in noradrenaline content between control animals and denervated umals is in no case statistically significant by the Student t test The great t difference is seen in the guinea pig vas deferens, where it is $2\,0 \pm 0\,92$

Table III Noradrenaline (NA) and adrenaline (A) in vas deferens and accessory male glands of the macaque and the effect of hypogastric denervation

Macaque no	Body weight (kg)	Weight of pair of vasa def (g)	NA in vas def (μg/g tissue)	A in vas def (μg/g tissue)	Weight of cran prost (g)	NA in cran prost (μg/g tissue)
Controls						
1	6.1	0.46	16.5	13.2	1.30	4.1
2	4.2	0.30	13.7	21.9	1.20	4.0
3	3.6	0.25	17.9	0.9	1.77	6.0
4	2.3	0.26	18.4	1.5	0.75	4.8
Mean	4.1	0.32	16.6	9.4	1.26	4.7
Hypogastric den 2 days						
5	2.7	0.26	16.2	1.2	0.70	4.1

μg/g tissue, but still not significant There are no significant differences between control groups and denervated groups in other respects, e g in body weights and weights of the *vasa deferentia* and the accessory male genital glands The dog prostates showed too much variation for statistical analysis This is due to the high incidence of prostatic hypertrophy in this species Yet there does not seem to be any reduction in the noradrenaline content after hypogastric denervation, as judged from a gross comparison of the material and by comparing littermates in the two groups

Since the noradrenaline content of the organs could be due to adrenergic nerves having other paths than the hypogastric nerves, pelvic denervation was performed on two dogs and total lumbosacral sympathectomy on six cats The results are seen in Table IX and X and in Figs 21 and 22 Pelvic denervation probably does not alter the noradrenaline content of the vas deferens and the prostate After lumbosacral denervation there is a decrease in the noradrenaline content of the organs The decrease in the vas deferens is significant (0 05 > P > 0 01)

The bulbourethral glands proved to have a very low content of catechol amines. They were therefore not so extensively studied The data obtained from different mammals are given in Table XIV

Most organs investigated showed uncertain values of adrenaline If present

Table III Continued

Macaque no	A in cran prost (µg/g tissue)	Weight of caud prost (g)	NA in caud prost (µg/g tissue)	A in caud prost (µg/g tissue)	Weight of pair of sem ves (g)	NA in sem ves (µg/g tissue)	A in sem ves (µg/g tissue)
Controls							
1	5 9	1 99	41 3	132 0	7 11	2.3	0 8
2	7 3	1 66	58 2	212 0	5 90	2 6	4.5
3	2 7	1.50	31 4	64 0	4 60	2 9	0 2
4	5 8	0 68	11 0	29 9	2 65	7 4	0 3
Mean	5 4	1 46	35.5	109.5	5 07	3 8	1.5
Hypogastric den. 2 days							
5	4 3	0 94	12 0	229 0	2 00	5 6	0 2

at all it constituted less than 5 per cent of the total catecholamines In the organs of the macaque, some dog prostates and one hedgehog prostate certain amounts were however found They are presented in Table II, III, and IX

DISCUSSION

It is concluded from the data presented in this chapter, that the vas deferens and the accessory male glands of different mammals contain large amounts of noradrenaline In addition to noradrenaline adrenaline has been found in some of these organs, thus it is present in all accessory male organs of the macaque, especially the prostate, where amounts of 200 µg/g tissue may be present Adrenaline may also be found in the prostate of the dog (and the hedgehog)

The noradrenaline content of the organs is not overtly reduced by section and degeneration of the hypogastric nerves (monkey, rabbit, guinea-pig, rat, dog, cat and sheep), nor is it reduced by pelvic denervation (dog) After total lumbosacral sympathectomy the noradrenaline content of the cat vas deferens is decreased, but still large amounts remain

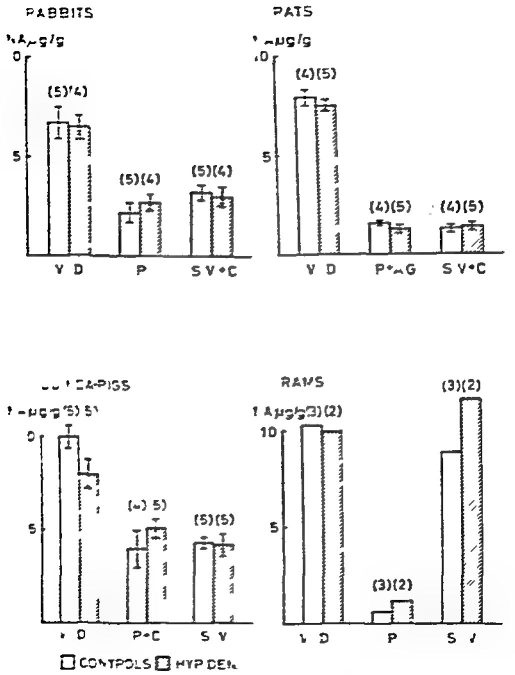


Fig 20 Effect of hypogastric denervation on the noradrenaline contents of the vas deferens, prostate and seminal vesicle of the rabbit, the rat, the guinea-pig and the ram. Means ± S.E.M. Controls white columns, hypogastric denervated shaded columns. Number of animals within brackets. V.D = vas deferens. P = prostate. C = coagulating gland, A.G = ampullary gland, S.V = seminal vesicle

Table II Noradrenaline (NA) in vas deferens of man Weights within brackets indicate weights of pieces used for analysis

Age (years)	Weight of vas def (g)	NA in vas def (µg/g tissue)
59	(0 12)	1 83
61	(0 12)	1 60
67	(0 25)	0.50
70	(0 10)	0 70
79	(0 10)	1 27

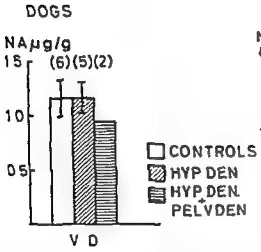


Fig 21 Effect of hypogastric denervation and pelvic denervation on the noradrenaline content of the dog vas deferens (means ± S E M) Number of dogs within brackets

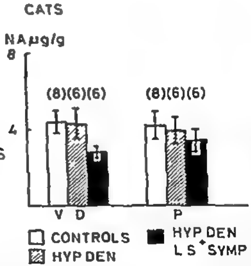


Fig 22 Effect of hypogastric denervation and lumbosacral sympathectomy on the noradrenaline content of the vas deferens and the prostate of the cat (means ± S E M) White columns controls shaded columns hypogastric denervated animals black columns animals subjected to hypogastric denervation and total lumbosacral sympathectomy Number of animals within brackets V D = vas deferens P = prostate

Table 1 Noradrenaline (NA) in vas deferens and accessory male glands of the rabbit and the effect of hypogastric denervation

Rabbit no	Body weight (kg)	Weight of pair of vasa def (g)	NA in vas def (µg/g tissue)
Controls			
1	1.9	0.26	9.4
2	2.0	0.40	5.8
3	2.9	0.64	5.4
4	2.2	0.31	7.2
5	2.7	0.48	5.5
Mean ± S.E.M	2.3±0.2	0.42±0.07	6.7±0.8
Hypogastric den			
6	2.4	0.36	7.2
7	3.2	0.71	4.6
8	2.4	0.43	7.2
9	2.2	0.62	7.0
Mean ± S.E.M	2.6±0.2	0.53±0.08	6.5±0.6

There seems to be a gross correlation between noradrenaline content and the amount of smooth muscle cells in accessory male organs, thus organs with rather thin smooth muscle layers, such as the prostates of the hedgehog (especially prostate I) and the seminal vesicle of the boar, have a rather low content of noradrenaline compared with the rest of the examined organs. The disseminated prostates of the artiodactyles, where the smooth muscle is mixed with other tissues in the stroma of the *urethra*, have also a rather low content of noradrenaline. Hyperplastic dog prostates have a lower content than normal prostates. (Prostatic enlargement in the dog is essentially due to cystic hyperplasia of the epithelium and does not involve muscular hypertrophy (*cf* Price and Williams-Ashman 1961).) Further the accessory male glands of the rat have a lower noradrenaline content than homologous organs in the guinea pig and the rabbit (*cf* Chapter II). Finally the bulbo-urethral glands mainly supplied by striated muscles, have a very low noradrenaline content. The highest content is generally seen in the vas

Table 1 Continued

Rabbit no	Weight of prostate (g)	NA in prostate (μg/g tissue)	Weight of seminal vesicle and coag gland (g)	NA in seminal vesicle and coag gland (μg/g tissue)
Controls				
1	0.47	3.8	0.49	4.5
2	0.59	1.1	0.63	2.8
3	0.41	1.8	1.03	2.6
4	0.29	1.6	0.40	2.6
5	0.56	2.8	0.61	3.5
Mean ± S E M	0.46±0.05	2.2±0.5	0.63±0.11	3.2±0.4
Hypogastric den				
6	0.32	3.8	0.43	4.0
7	0.36	3.0	0.72	2.2
8	0.41	2.5	1.20	3.7
9	0.71	1.6	0.84	2.1
Mean ± S E M	0.45±0.09	2.7±0.4	0.80±0.16	3.0±0.5

deferens, which has a relatively more prominent muscle layer than the accessory genital glands In this respect there are, however, two peculiar exceptions the *vasa deferentia* of the dog and its relative, the fox, which have comparatively low noradrenaline contents (about 1 μg/g tissue) It is difficult to draw definite conclusions regarding the actual level of noradrenaline in the human vas deferens from the present material, for there is too wide

Table 1.1 Noradrenaline (NA) in coagulating gland and seminal vesicle of the rabbit

Rabbit no	Body weight (kg)	Weight of coagulating gland (g)	NA in coag gl (μg/g tissue)	Weight of seminal vesicle (g)	NA in sem vesicle (μg/g tissue)
10	2.3	0.30	2.4	0.18	5.7
11	3.4	0.52	1.4	0.40	7.5
12	2.8	0.21	2.5	0.31	5.5
Mean	2.8	0.34	2.1	0.30	6.2

Table VII Noradrenaline (NA) in vas deferens and accessory male glands of the rat and the mouse and the effect of hypogastric denervation

Rat no	Body weight (g)	Weight of pair of vasa def (g)	NA in vas def (μg/g tissue)
Controls			
1	245	0.12	6.8
2	250	0.13	8.6
3	260	0.09	8.0
4	240	0.10	8.3
Mean ± S.E.M.	249±4	0.11±0.01	7.9±0.4
Hypogastric den			
5	255	0.12	7.5
6	240	0.09	8.0
[illegible]	245	0.08	8.0
8	240	0.10	7.5
9	250	0.11	6.5
Mean ± S.E.M.	246±3	0.10±0.01	7.5±0.3
Group of mice no			
1		0.05	4.4
2		0.08	5.5
3		0.06	6.3
Mean			5.4

a range in the values; further, the *vasa deferentia* are from old men operated for prostatic hypertrophy or cancer of the prostate and finally the tissue pieces were not extracted immediately after removal. (It should, however, be noted that the highest value was obtained in the only case having spermatozoa in the vas deferens.)

With the exception of chromaffin tissue and adrenergic ganglia, no peripheral mammalian tissue hitherto examined contains such large amounts of noradrenaline as the vas deferens and the accessory male glands (excepting the bulbo-urethral glands). These organs often contain 5–10 times as much noradrenaline as do the spleen and the heart in the same species (for reference values *cf.* Euler 1956 and 1963). The comparatively high amounts of adrenaline in the accessory male organs of the macaque should also be

Table V II Continued

Rat no	Weight of prostate + ampullary glands (g)	NA in prostate + amp glands (µg/g tissue)	Weight of pair of sem vesicles and coag glands (g)	NA in sem. vesicle and coag gland (µg/g tissue)
Controls				
1	0.79	1.21	0.31	0.88
2	0.64	1.63	0.43	1.18
3	0.39	1.63	0.29	1.58
4	0.48	1.80	0.36	1.38
Mean ± S E M	0.58±0.09	1.57±0.13	0.35±0.03	1.26±0.15
Hypogastric den				
5	0.49	0.87	0.34	1.34
6	0.60	1.16	0.41	1.03
7	0.32	1.85	0.20	1.87
8	0.44	1.04	0.31	1.22
9	0.48	1.46	0.37	1.59
Mean ± S E M	0.47±0.05	1.28±0.17	0.33±0.04	1.41±0.15
Group of mice no				
1			0.57	0.83
2			0.65	1.14
3			0.72	0.99
Mean				0.99

noted since most mammalian organs except the adrenals contain no or only sparse amounts of this catecholamine

According to the fundamental studies of Euler and his coworkers (*cf* Euler 1956 and 1961) tissue noradrenaline levels reflect the degree of adrenergic innervation of the tissues The present results then indicate a very rich adrenergic innervation of the vas deferens and the accessory male glands (except the bulbo-urethral glands) Anticipating the next chapter (Chapter VI) there seems, however, not always to be an exact correlation between the noradrenaline content of a tissue and the distribution of adrenergic terminals in it For example, the rat vas deferens appears to have more terminals than the guinea pig vas deferens Yet the latter has a higher noradrenaline content than the former This may be due to differences in the development

Table I:III Noradrenaline (NA) in vas deferens and accessory male glands of the guinea pig and the effect of hypogastric denervation

Guinea-pig no.	Body weight g	Weight of pair of vas def. (g)	NA in vas def. (μg/g tissue)	Weight of pair of seminal ves. (g)	NA in sem. ves. (μg/g tissue)
Controls					
1	380	0.11	10.0	0.42	5.2
2	610	0.16	9.7	0.94	5.1
3	485	0.16	11.2	0.65	4.0
4	520	0.18	8.7	0.86	3.6
5	450	0.10	11.8	0.49	4.1
6	560	0.16	8.3	0.63	3.9
Mean ± S.E.M.	501±33	0.15±0.01	10.0±0.6	0.67±0.08	4.3±0.3
Hypogastric den.					
7	395	0.10	6.8	0.50	6.0
8	500	0.16	6.4	0.88	3.3
9	455	0.12	7.4	0.46	4.4
10	515	0.13	10.5	0.55	4.4
11	550	0.15	8.9	0.84	2.8
Mean ± S.E.M.	483±27	0.13±0.01	8.0±0.8	0.65±0.09	4.2±0.6

of preterminal varicosities (see Chapter VI) and perhaps also to species variations in concentration of the transmitter in the terminals. A further discussion of the relation between noradrenaline content and adrenergic innervation is given in Chapter VI.

The relatively low noradrenaline content of the dog—and fox—vas deferens is puzzling, since even nonmammalian vasa deferentia (Sjöstrand 1965 and Fig. 17) have been found to contain large amounts of noradrenaline. This peculiar finding will, however, be discussed in detail in the following chapter.

The persistence of the tissue noradrenaline content after section and degeneration of the main sympathetic nerves (the hypogastric nerves) to the investigated organs indicates that these organs are chiefly innervated by short adrenergic neurons located close to the target organs. Thus adrenergic nerve

Table VIII Continued

Guinea pig no	Body weight (g)	Weight of prost and coag gl (g)	NA in prost and coag gl (μg/g tissue)		
Controls					
12	510	0.42	1.6		
13	475	0.36	5.5		
14	580	0.24	3.4		
15	420	0.58	5.6		
Mean ± S.E.M.	496±34	0.40±0.07	4.0±1.0		
Hypogastric den					
16	410	0.34	3.7		
17	470	0.27	5.1		
18	505	0.47	5.7		
19	445	0.30	6.4		
20	415	0.29	4.6		
Mean ± S.E.M.	449±18	0.33±0.04	5.1±0.5		

cells located in the inferior mesenteric ganglion do not in any appreciable degree take part in the adrenergic innervation of the vas deferens and the accessory male glands. The reduction in noradrenaline content seen in the vasa deferentia of the cats submitted to resection of the lumbosacral sympathetic chain is probably due to a reduction in vascular innervation (*cf.* Chapter VI). In this respect it should be noted that the cat vas deferens has a more prominent vascular supply in its surrounding connective tissue than the other examined species (*cf.* Chapter VI). Since first reported (Sjöstrand 1962 b) the persistence of the noradrenaline content of the guinea pig vas deferens after hypogastric denervation has been confirmed by Ohlin and Strömblad (1963).*

* The noradrenaline content of the guinea pig vas deferens reported by Ohlin and Strömblad (1963) is considerably lower than that found by me (Sjöstrand 1962 and Table VIII in this report), namely 2.34 μg/g tissue wet weight. Moreover they found adrena

Table XI Noradrenaline (NA) in vas deferens and accessory male glands of the boar. Weights within brackets indicate weight of pieces used for analysis

Boar no	Weight of vas def (g)	NA in vas def (µg/g tissue)	Weight of prost body (g)	NA prost body (µg/g tissue)	Weight of diss prost (g)	NA diss prost (µg/g tissue)	Weight of sem ves (g)	NA in sem ves (µg/g tissue)
1	(0.14)	4.6	(0.90)	2.4	(0.90)	0.92	(1.00)	0.88
2	(0.[illegible]7)	5.8	(1.80)	2.9	—	—	(2.10)	1.30
3	(0.51)	9.7	(1.62)	1.8	(1.52)	0.75	(1.99)	1.01
Mean		6.7		2.4		0.84		1.06

line 0.41 µg/g tissue—in the vas deferens. This discrepancy is puzzling because two control expts performed by me using the same method as they reported (that of Bertler, Carlsson and Rosengren (1958) and with pooled vasa deferentia from 4 guinea pigs in each determination yielded 9.9 and 11.0 µg noradrenaline/g tissue wet weight and no certain amounts of adrenaline.

In this connection the results of Burnstock and Holman (1962) are of interest. They found that after section of the hypogastric nerve, stimulation of the distal part still could elicit junction potentials in the smooth muscle cells of the guinea pig vas deferens. Further they also found that there still was a discharge of spontaneous small potentials in the vas deferens after denervation although the rate of discharge was much slower in the denervated

Table XII Noradrenaline (NA) in vas deferens and accessory male glands of the bull. Weights within brackets indicate weight of pieces used for analysis

Bull no	Weight of vas def (g)	NA in vas def (µg/g tissue)	Weight of amp vas def (g)	NA in amp vas def (µg/g tissue)	Weight of prost. body (g)
1	(0.20)	7.0	(1.25)	15.0	(1.17)
2	(0.10)	13.1	(0.96)	17.6	(0.80)
3	(0.[illegible])	7.7	(1.40)	9.5	(0.92)
Mean		9.3		14.0	

preparations than in nondenervated ones. It might be, however, that the "spontaneous" discharge in nondenervated preparations is fascilitated by intact presynaptic fibres (or due to activity in their cut ends). For further discussion see Sjöstrand 1962 b.

According to the suggestion of Euler (*cf* Euler 1956 and 1961) adrenaline in peripheral tissue is due to the presence of chromaffin cells. Again anticipating the following chapter (Chapter VI), it could be stated that the adrenaline present in the investigated organs probably is stored in chromaffin cells. Thus such cells are abundantly found in the accessory male organs of the macaque and they are also seen in the dog prostate. The wide range seen in the adrenaline contents of the organs of the macaque compared to those of noradrenaline may also indicate a storage in chromaffin cells since the number of chromaffin cells in the organs may vary much more than the degree of adrenergic innervation of them and noradrenaline is probably mainly stored in adrenergic terminals. However, it is likely that some chromaffin cells contain noradrenaline as for instance those of the cat prostate (*cf* Chapter VI) and part of the noradrenaline content of the macaque organs is probably stored in chromaffin cells. This would explain the comparatively high noradrenaline content in the vas deferens and the caudal prostate of the macaque. In this respect the high content of noradrenaline in the vas deferens of the tortoise should be noted. In this animal, there is a very close relation between the adrenals and the vas deferens.

Some species (hedgehog, mouse, fox, pig, bull and sheep) were not used for histochemical analysis. From a comparison between their noradrenaline values and those of the species used in the next chapter, it seems, however, likely that at least the major part of the noradrenaline in their accessory male organs is stored in adrenergic terminals and not in chromaffin cells.

Table XII Continued

Bull no	NA in prost body (µg/g tissue)	Weight of diss prost (g)	NA in diss prost (µg/g tissue)	Weight of sem. ves. (g)	NA in sem. ves. (µg/g tissue)
1	6.1	(1.17)	0.22	(1.51)	8.0
2	11.3	(2.09)	1.02	(2.24)	13.5
3	9.2	(1.70)	1.54	(1.50)	10.7
Mean	8.8		0.93		10.7

Table VIII Noradrenaline (NA) in vas deferens and accessory male glands of the ram and the effect of hypogastric denervation

Ram no	Body weight (kg)	Weight of pair of vasa def + amp vas def (g)	NA in vas def + amp vas def (µg/g tissue)	Weight of prost (g)	NA in prost (µg/g tissue)	Weight of pair of sem ves (g)	NA in sem ves (µg/g tissue)
Controls							
1	26	1.92	10.4	4.79	0.37	2.87	8.4
2	21	1.29	14.7	4.10	0.33	2.14	10.2
3	20	[illegible]21	[illegible]	2.82	1.09	3.36	8.1
Mean	[illegible]3	1.81	10.3	3.90	0.56	2.79	8.9
Hypogastric den							
4	27	2.11	10.6	2.93	0.94	2.75	13.4
5	22	2.02	9.4	2.10	1.38	1.81	10.1
Mean	25	2.07	10.0	2.54	1.16	2.28	11.8

Table XII Noradrenaline (NA) in Cowper's glands of some mammals

Species	Organ weight (g)	NA (µg/g tissue)
Macaque	0.29	0.35*
Rabbit	0.21 0.30 0.34	0.19 0.02 0.09
Cat	0.20 0.27 0.25	0.38 0.00 0.00
Boar	191)	0.09
Ram	0.70 0.58	0.00 0.00

* Adrenaline 0.25 µg/g tissue

CHAPTER VI

CELLULAR LOCALIZATIONS OF CATECHOLAMINES IN ACCESSORY MALE GENITAL ORGANS

In chapter V it was concluded that the vas deferens and the accessory male glands of different mammals contained large amounts of noradrenaline and in some species also adrenaline The fluorescence microscopic method reported by Falck (*cf* Chapter III) has made the direct demonstration of cellular catecholamines possible By means of this method an abundant occurrence of adrenergic terminals was demonstrated in the guinea pig vas deferens (Falck 1962), corresponding well to the simultaneous finding of the large content of noradrenaline in this organ (Sjostrand 1962 b) Furthermore Falck (1963) observed adrenergic nerve cells in the distal part of the hypogastric nerve of the guinea pig

Consequently the next step in this investigation was to achieve further information about the catecholamine containing structures present in the vas deferens and the accessory male genital organs of different species The work constituting this part of the investigation has also been included in two separate reports (Falck, Owman and Sjostrand 1965, Owman and Sjostrand 1965)

RESULTS

The guinea pig was chosen as "reference animal Therefore in this chapter the species are grouped after the number of animals used and not after the common zoological classification scheme used in the previous chapters

Guinea pig

In the normal *vas deferens* an abundance of intensely green fluorescent varicose nerve terminals was found in a very dense plexus within both the circular and longitudinal muscle layers The terminals ran along the muscle

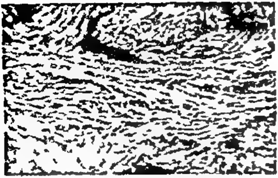


Fig 23 Seminal vesicle of the rabbit Longitudinal section The figure is an example of the very dense adrenergic innervation seen in most internal male genital organs of different species. 103 X

cell chiefly following their direction (cf Fig 23) Thus in the middle layer the major part of the varicose terminals ran circularly, and in the inner and outer layers they ran longitudinally In the peripheral parts, and just outside the vas deferens several nerve bundles were present, containing mainly intensely fluorescent varicos fibers but also smooth preterminal axons emitting a weaker fluorescence Somewhat more proximally these bundles consisted almost entirely of smooth faintly fluorescent nerve fibers The bundles ran parallel to the vas deferens branching into small fascicles, which penetrated the wall to ramify in the organ Small radial bundles issued from the outer longitudinal layer into the circular one, where they contributed to the circular plexus In a similar manner the plexus of the inner longitudinal layer received its supply from the circular layer No clear difference in the amount of innervation could be seen comparing the distal and middle levels of the vas deferens but in the proximal section there was an over all tendency towards a decreasing density of the nerve plexuses

Sections from the *epididymis* showed that the heavy innervation started concomitantly with the appearance of a muscle layer The muscle innervation persisted along the entire extent of the *ejaculatory ducts* A sphincter like structure was not found Only rarely were fluorescent terminals seen in the mucosa and those present appeared to accompany minute vessels rather than serving the mucosal cells Outside and running along the vas deferens were seen a great number of blood vessels surrounded by rather dense plexa of adrenergic nerves superimposing upon but not or only to a very small extent penetrating the media (cf Falck 1962)

The distribution pattern of fluorescent nerves in the *seminal vesicle* was similar to that of the vas deferens although it was not so extensively innervated as the latter organ

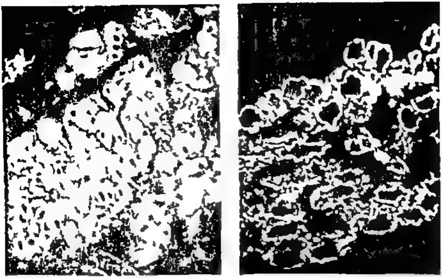
The *prostates* and the *coagulating gland* were found to have an innerva

tion of green fluorescent terminals very similar to that seen in the seminal vesicle, the coagulating gland having in its thicker muscle coat a somewhat richer innervation than the prostates In young animals both organs showed a slightly thicker muscle layer and a corresponding richer innervation than those of older animals In the seminal vesicle, the prostate and the coagulating gland the density of fluorescent nerves was the same along the whole extent of the organ On the other hand there was a tendency toward decreasing thickness of the muscle layer in the proximal parts

In the terminal parts of the hypogastric nerve, where it joins the vessels to the accessory genital organs and ramifies masses of nerve cell bodies occurred, generally arranged in clusters (Figs 24 A and 27) The nerve cells were rather uniform in size and showed varying degrees of fluorescence from very faint to quite high intensities Among the fluorescent cells groups of completely non fluorescent nerve cells occurred Their presence could be registered due to a very faint unspecific background fluorescence but appeared clearly when the sections were studied by phase-contrast microscopy Around some of the cells, both of the fluorescent and the non fluorescent types adrenergic terminals were seen However, the majority of cells did not receive any terminals Scattered in these ganglia were located small intensely green yellow to yellow fluorescent cells, isolated or more frequently collected in groups Several long and branching processes extended from these cells Leaving the peripheral ganglia bundles of smooth moderately green fluorescent as well as non fluorescent axons could be seen In serial sections these bundles were seen to branch and form the peripheral bundles outside the vas deferens and the accessory genital glands Fluorescent as well as non-fluorescent nerve cells were also dispersed over a rather wide area in the vicinity of the accessory male genital glands, and were regularly seen in the walls of the prostate and the coagulating gland too (Fig 27) They could not be found within the vas deferens or in the seminal vesicle

No visible reduction of the peripheral innervation was observed in structures from the side where the hypogastric nerve was cut, as compared with the non-denervated side or with unoperated control animals Nor was there any reduction in the vascular innervation nor a decrease in the amount of adrenergic nerve terminals around the peripheral nerve cells

Rat The *vas deferens* of the rat showed an innervation quite similar to that of the guinea pig except that the fluorescent nerve fibers appeared to be more delicate and somewhat more frequent The nervebundles running outside the organ consisted mainly of moderately green fluorescent smooth fibers whereas intensely fluorescent varicose fibers occurred only in small numbers



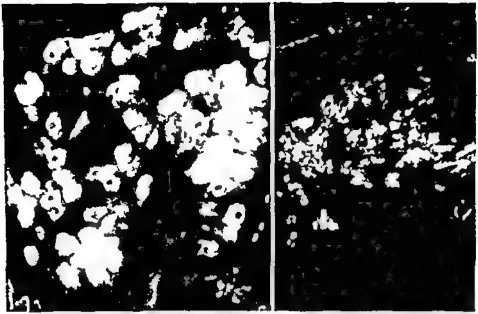
A B

Fig. 24. Peripheral adrenergic ganglia located near the internal male genital organs of the guinea pig and the rabbit.

A. Ganglion located in the wall of the coagulating gland of the guinea pig. Nerve cells showing varying degree of green fluorescence. Among the fluorescent cells some cells lacking fluorescence are seen. Note the highly fluorescent varicose terminals around some nerve cells. 115 ×.

B. Ganglion located near the seminal vesicle of the rabbit. The nerve cells display almost no fluorescence but are surrounded by smooth fluorescent nerve fibers. 115 ×.

The *accessory male genital glands* of the rat had a sparser innervation than the vas deferens in their rather thin muscle coats. The thickest muscle layer and the largest amount of fluorescent terminals was found in the coagulating gland, followed by the seminal vesicle and the ampullary glands. Parts of the parenchymatous prostate, especially the ventral lobes with their thin muscular capsules and delicate septa, contained rather few fluorescent terminals. The dorso-lateral groups of acini generally showed thicker muscle walls and a greater amount of fluorescent varicose fibers. The fluorescent terminals even in the accessory male genital glands seemed to be restricted to the smooth muscle tissue (and the blood vessels) and did not seem to innervate the parenchymal cells. In the distal part of the hypogastric nerve clusters of green fluorescent and non-fluorescent nerve cells similar to those of the guinea pig were seen (Figs. 25 A and 28), the main difference being that fluorescent varicose terminals only very rarely occurred around the nerve cells of the rat. The same types of nerve cells were also observed in the wall



A B

Fig 25 Peripheral adrenergic ganglia located near the internal male genital organs of the rat and the macaque

A Ganglion located near vas deferens of the rat Nerve cells showing varying degree of fluorescence Among the fluorescent cells some cells devoid of fluorescence are located From the ganglion a bundle of faint fluorescent smooth nerve fibers is leaving In the lower right corner of the figure some mastcells are seen 115 X

B Ganglion located in the connective tissue sheath of the seminal vesicle of the macaque Small nerve cells displaying varying degree of fluorescence are seen Note the small size of the nerve cells when compared with those of the rat (and the guinea pig and rabbit Fig 24) 115 X

of the ampullary gland in the bases of the coagulating gland and the seminal vesicle as well as in those peripheral parts of the prostate located in the vicinity of the hypogastric nerve (Fig 28)

As in the guinea pig no change in the amount of fluorescent nerve fibers in the organs could be seen following hypogastric denervation

Rabbit The *vas deferens* showed the same pattern of innervation as in the guinea pig and the rat but the meshes of the fluorescent terminals appeared somewhat wider No clear cut difference in the density of fluorescent terminals could be observed at the various levels of the rabbit vas deferens except that since this animal possesses a distinct ampullary part with a thinner

Fig. 26 Cluster of small intensely fluorescent cells in the prostate of the dog 115 X
A Section mounted in liquid paraffin The flourophore is confined to the cell group
B Section mounted in xylene containing entellan Note the diffusion of the fluorophore indicating that adrenaline is present in the cells

muscular coat the amount of fluorescent nerves is less in this part than in the rest of the vas deferens

The sacculous *seminal vesicle* which in contrast to that of the guinea pig and the rat has a quite prominent outer longitudinal muscle layer, showed a dense innervation of its muscularis (Fig 23), the innervation density being almost more pronounced than in the vas deferens

The muscle layers of the multitubular *coagulating* and *prostate glands* showed almost the same density of adrenergic innervation as corresponding organs in the guinea pig the coagulating gland appeared to have a somewhat heavier innervation than the prostate

Peripherally located *nerve cell bodies* were found in the near vicinity of the internal accessory male genital organs and in the walls of the seminal vesicle Fig 29) They were closely packed to form several ganglia distributed within a rather wide area in this region The cell bodies emitted no or only a faint green fluorescence which in cases was so weak that it could not be established whether it was a specific fluorescence or only an unspecific background fluorescence Close around the cells of some groups was seen a peculiar arrangement of large numbers of moderately green fluorescent smooth fibers running in an irregular meshwork (Fig 24 B) In other groups several intensely green fluorescent varicose terminals ran contiguous to the

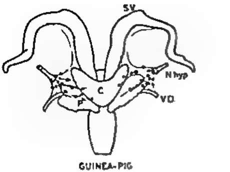


Fig 27 Schematic drawing illustrating the localization of the adrenergic ganglia innervating the internal male genital organs of the guinea pig in relation to these organs Dorsal view V D = vas deferens S V = seminal vesicle P = prostates C = coagulating gland N hyp = hypogastric nerve Adrenergic ganglia indicated by black dots

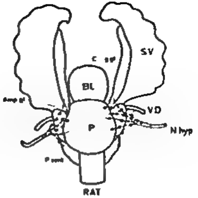


Fig 28 Schematic drawing illustrating the localization of the adrenergic ganglia innervating the internal male genital organs of the rat in relation to these organs The lateral group of prostatic acini is separated from the dorsal The ampullary glands located in front of the seminal vesiclesand the coagulating glands are indicated by dotted lines Dorsal view V D = vas deferens S V = seminal vesicle P = prostate Amp gl = ampullary glands P ventr = ventral prostate N hyp = hypogastric nerve Adrenergic ganglia indicated by black dots

nerve cells, and finally there were accumulations of cells among which no or only few adrenergic terminals were observed The ganglia also contained scattered, small multipolar cells emitting an intense green yellow to almost orange fluorescence as was the case in the guinea pig and the rat

In rabbits treated with nialamide and l DOPA a diffuse moderate green fluorescence appeared throughout the tissues However, the green fluorescence of the nerve terminals was considerably increased, so that they now exhibited a yellowish glare, and the small branching cells emitted an even more intense yellow orange light In the ganglia it was clearly evident that the cells surrounded by smooth fibers (see above) emitted a very intense yellow green fluorescence, while the rest remained only with the low background fluorescence Thus, there were obviously three types' of cells in these ganglia a) green fluorescent nerve cells, surrounded by the smooth proximal parts of the axons from nearly located cells, these axons finally collect to

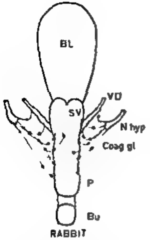


Fig 29 Schematic drawing showing the localization of the adrenergic ganglia innervating the internal male genital organs of the rabbit in relation to these organs Dorsal view V.D = vas deferens S V = seminal vesicle Coag gl = coagulating gland P = prostate N hyp = Hypogastric nerve Adrenergic ganglia indicated by black dots

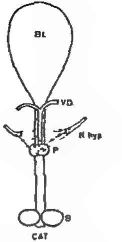


Fig 30 Schematic drawing showing the localization of the adrenergic ganglia innervating the vas deferens and the prostate of the cat in relation to these organs Dorsal view V D = vas deferens P = prostate N hyp = hypogastric nerve Adrenergic ganglia indicated by black dots

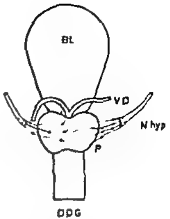


Fig 31 Schematic drawing showing the localization of the adrenergic ganglia innervating the vas deferens and the prostate of the dog in relation to these organs Dorsal view V D = vas deferens P = prostate N hyp = hypogastric nerve Adrenergic ganglia indicated by black dots

form the postganglionic adrenergic nerve bundles (Fig 24 B) b) non fluorescent nerve cells surrounded by several adrenergic terminals and c) non fluorescent nerve cells surrounded by some few fluorescent terminals

As in the guinea pig and the rat hypogastric denervation had no effects on peripheral fluorescent structures

Cat The *vas deferens* of the cat showed an innervation by green fluorescent terminals of principally the same arrangement as that of the rabbit In comparison with the rodents examined the outer and inner longitudinal muscle layers were quite thin while the circular one was much thicker The circular and the inner longitudinal layers possessed a somewhat richer adrenergic innervation than the outer longitudinal coat An abundance of rather heavily innervated blood vessels ran outside the vas deferens In the funicular parts of the organ the innervation of the muscle layers was more

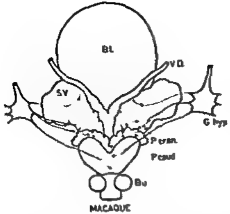


Fig 32 Schematic drawing showing the localization of the adrenergic ganglia innervating the internal male genital organs of the macaque in relation to these organs Dorsal view V D = vas deferens S V = seminal vesicle P cran = cranial prostate P caud = caudal prostate G hyp = hypogastric ganglion Adrenergic ganglia indicated by black dots

sparse than in the abdominal parts The preterminal nerve bundles running in large number outside the vas deferens consisted mainly of smooth faintly green fluorescent fibers, while varicose fibers were rare

The *prostate* of the cat possesses a strong muscle coat and thick septa which were abundantly innervated by adrenergic terminals running along the muscle cells Green yellow to almost orange fluorescent cells of the small polymorph type often sending out several long processes in the muscle layer, lay scattered in the prostatic muscle tissue In contrast to most of the small cells of similar morphology seen in other species (see below) those cells in the prostatic wall of the cat showed no fluorescent halo when mounted in Entellan (*cf* Fig 26 A, B)

In the terminal parts of the hypogastric nerve, and within the connective tissue enclosing the prostate and the proximal parts of the vas deferens were seen several groups of nerve cells (*cf* Fig 30) These clusters consisted either of only non fluorescent rather big nerve cells or of a mixture of such cells and green fluorescent nerve cell bodies, the latter cells dominating in number The fluorescent cells showed great variation in size Generally, the smaller cells exhibited a stronger fluorescence than the bigger ones Many of the fluorescent, but few of the non fluorescent nerve cellbodies were surrounded by green fluorescent varicose terminals Fluorescent preterminal bundles running outside the vas deferens and the prostate could be traced back to clusters containing fluorescent nerve cells In the vicinity of, or sometimes within the ganglia were seen groups of the small, intensely green yellow fluorescent branching cells many of which showed a diffusion of the fluorophore after mounting in Entellan

Neither hypogastric nor total lumbosacral denervation caused any visible reduction in the density of fluorescent terminals in the vas deferens and the

prostate However following lumbosacral denervation there was observed a marked decrease in the amount of innervation of the blood vessels in these organs although they were not completely devoid of fluorescent nerves No decrease in fluorescent terminals around the peripheral ganglion cells could be noted with these types of denervation In that animal in which an attempt was made to remove the peripheral ganglia on the right side, there occured a moderate decrease in the number of adrenergic fibers in the right prostatic lobe, as compared to the left

There was also a clear and apparently equal decrease in the amount of nerve supply to the vas deferens of both sides indicating a crossing-over of terminals from the ganglia of both sides

Dog The *vas deferens* of the dog differed markedly from that in the other species examined The outer longitudinal coat was very sparsely innervated by adrenergic terminals The same was the case with the thick circular layer The only layer possessing a degree of innervation like that seen in the other species was the inner longitudinal layer With silver staining it could, however, be demonstrated that the circular layer possesed a dense supply of nerves that apparently are not adrenergic The vessels of the vas deferens showed a normal adrenergic innervation

The *prostate* of the dog had a rather dense supply of adrenergic terminals in its capsule which was thicker in the dorsal lobes than in the ventral one The same rich nerve supply was seen in the septa of the dorsal lobes, while the ventral lobes being largely parenchymatous and having but few smooth muscle fibers, had a sparse innervation similar to that seen in the ventral prostate of the rat In the septa of the dorsal lobes, small cells with branching processes were seen They often occurred very closely packed in large groups and exhibited an enormous fluorescence making it difficult to outline clearly the individual cells (Fig 26 A) In contrast to the similar cells in the cat most of them showed a considerable diffusion of the fluorophore when mounted in Entellan which, however, was not seen after mounting in liquid paraffin (Fig 26 B) Moreover they exhibited a more intense fluorescence when treated in formaldehyde for three hours, as compared with one hour s treatment Many of these cells were situated in the nerve plexa, their processes intermingling with the nerve fibers No fluorescent nerve cell bodies were seen within the glandular or muscular parts of the dog prostate

In the connective tissue adjacent to the prostate (and in its sheats) and the vas deferens where the hypogastric and the pelvic nerve form plexa, groups of ganglion cells were seen (Fig 31) Fluorescent nerve cells were not as frequent as non fluorescent ones and in pieces taken from the plexus

between the bladder and rectum they were very scanty As in the cat the fluorescent cells varied markedly in size, the smaller ones generally having a stronger green fluorescence Fluorescent terminals were rather rare around the cell bodies and almost exclusively restricted to fluorescent cells

As to the peripheral innervation no difference could be seen whether the hypogastric nerves was cut or not

Macaque The *vas deferens* showed in contrast to the dog principally the same innervation pattern as in the other species investigated The inner part was, however, not so heavily innervated as the periphery but branching terminals were very frequent, perhaps because the innervation was not yet fully developed in these young macaques A tiny cluster of strongly yellowish fluorescent small cells with branching processes was observed in the connective tissue outside the vas deferens

The convoluted *seminal vesicles* were not so densily supplied by adrenergic terminals as the vas deferens Small groups of the intensely fluorescent polymorph cells were observed in the wall and in the interior of the organ The fluorescence was generally more intense in preparations treated for three hours with formaldehyde than in those treated for only one hour In sections mounted in Entellan the cells were surrounded by a wide, green fluorescent halo, which was not present in sections, mounted in liquid paraffin

The multitubular cranial lobe of the *prostate* gland had a rich supply of fluorescent terminals in the rather heavy muscle layer The preterminal bundles running outside this and the other organs seemed to consist of only smooth, faintly green fluorescent fibers Tiny groups of the strongly fluorescent small cells occurred more frequently in this organ than in the seminal vesicles In the caudal lobe of the prostate having an appearance with regard to muscular supply and lobation which resembles the human prostate, adrenergic terminals were seen in the muscular layer As in the vas deferens the innervation might not be fully developed Green yellow fluorescent small cells, often with branching processes and similar in shape and histochemical characteristics to those earlier described, lay scattered in the gland in great number and generally in small clusters

The *hypogastric ganglia* contained mainly large non fluorescent cells, only very few fluorescent nerve cells being present in them Green fluorescent, remarkably small nerve cell bodies (*cf* Fig 25 B) were, however, observed in very large number in the walls of the accessory genital glands, especially in the proximal part of the seminal vesicle (Fig 32) and the cranial lobe of the prostate, whereas comparatively few occurred in the caudal lobes The fluorescence intensity varied but strongly fluorescent cells predominated

The preterminal fluorescent bundles innervating the genital organs were seen to issue from these fluorescent nerve cells Among these cells lay scattered a few non fluorescent nerve cells A minority of the adrenergic nerve cells were surrounded by fluorescent terminals Small, intensely fluorescent branching cells were dispersed among the ganglion cells No ganglion cells were found within the musculature of the actual organs

The *bulbourethral* glands of the guinea pig and the rabbit were also studied These glands consists of a very thin smooth muscle layer surrounded by an abundance of striated muscle Besides vascular innervation a sparse amount of fluorescent terminals was observed in the thin smooth muscle layer only

The presence of enterochromaffin like cells in the epithelial linings of some accessory male glands and further the fluorescent structures in the inferior mesenteric ganglion and the hypogastric nerve are reported and discussed elsewhere (Owman and Sjostrand 1964) In that report a "model experiment on the chromaffin cells of the adrenal medulla of the guinea pig which contains almost entirely adrenaline, is described It was demonstrated that these cells behave histochemically like the majority of the small intensely fluorescent branching cells described here, i e they show a more intense fluorescence after prolonged treatment in formaldehyde, and the fluorophore diffuses when the sections are mounted in Entellan but not after mounting in liquid paraffin Further photomicrographs on the innervation of fluorescent terminals in the vas deferens and the accessory male glands are presented in the two reports on which this chapter is based (Falck, Owman and Sjostrand 1965, Owman and Sjostrand 1965)

DISCUSSION

It has been shown in the present investigation that the vas deferens and the accessory male glands of different animals possess an adrenergic innervation of a generally very high density No visible reduction in this innervation is seen following section of the hypogastric nerves The nerve cell bodies that send out the adrenergic axons forming the ground plexus of the internal accessory male genital organs are chiefly located in the close vicinity of the organs, within their walls or in the peripheral ramifications of the hypogastric nerves The adrenergic varicose terminals are present in rich amounts in these organs and are distributed in the smooth muscular coats and septa, as well as around the blood vessels No data indicating an adrenergic inner

vation of the secretory cells of these organs have been obtained The adrenergic vascular supply seems to derive chiefly from nerve cells located in lumbosacral parts of the sympathetic chain and partly from the adrenergic nerve cells close to the genital organs as judged from the results obtained in the cat Adrenergic fibers descend from the inferior mesenteric ganglia in the hypogastric nerves but do not seem to take any obvious part in the innervation of vessels and musculature of the internal male genital organs (Owman and Sjostrand 1965) The adrenergic nerve plexus of the vas deferens in the examined species the dog being an exception, is extremely dense indicating that a great part—in some species probably all—of the muscle cells receive adrenergic terminals most of the smooth muscle cells probably lying in close contact with more than one fiber Of the accessory genital glands, those having thick muscular coats receive rather abundant amounts of adrenergic terminals the innervation being sparser in organs largely composed of parenchymatous tissue, such as the ventral parts of the prostates of dog and rat In the dog vas deferens the adrenergic innervation is chiefly restricted to the internal longitudinal layer, the thick circular layer having a dense supply of non adrenergic axons The structure of the adrenergic innervation of these organs was found to agree completely with the construction of the autonomic ground plexus as described by Hillarp (1946, 1959) and the findings of Falck (1962) concerning the adrenergic plexus in different peripheral organs Thus, Richardson s (1962) findings that the nerve terminals in the vas deferens of the rat terminate with specialized endings supposed to be the important structure for nervous transmission finds no support in these investigations In this connection it should be mentioned that evidence now exists that the varicosities of autonomic nerves represents synaptic contacts (Elfvin 1963)

In the accessory male organs of the macaque, especially the prostate and in the muscle coat of the prostate of the dog and cat are seen small cells exhibiting an intense green yellow to almost orange fluorescence Numerous such cells are seen scattered among the adrenergic cell bodies located close to the internal genital organs and also in the inferior mesenteric ganglion and the hypogastric nerve (Owman and Sjostrand 1965) These small cells, often having branching processes generally show more intense fluorescence after prolonged treatment in formaldehyde In sections mounted in the presence of organic solvents they show a green halo of diffused fluorophore Thus they possess similar histochemical characteristics as the adrenal medullary cells of the guinea pig (Owman and Sjostrand 1965), known to contain almost only adrenaline, the fluorophore of which, in contrast to those of the primary catecholamines and 5 hydroxytryptamine, dissolves in certain or-

ganic solvents (*cf* Falck and Owman, 1965) Some of the small cells, however, do not show this halo and exhibit a fully developed fluorescence after a shorter exposure to formaldehyde In the cat prostate this type of small cell is the only one present The enormous fluorescence of these cells indicates that high amounts of catecholamine are stored in them and they may therefore well be chromaffin cells Hamberger, Norberg & Sjoqvist (1963) found similar cells in the inferior mesenteric ganglion and tentatively identified them as chromaffin cells Further, their occurrence in the accessory male genital organs corresponds well to the adrenaline levels in these organs (*cf* Chapter V) Thus, such cells are seen in the genital organs of the monkey, where adrenaline is found, especially in the prostate which holds considerable amounts of adrenaline, and they are seen in the dog prostate, where adrenaline also may be found The cells do not occur in the organs of rat, guinea-pig and rabbit, which do not contain measureable amounts of adrenaline In the cat prostate, on the other hand, the small, intensely fluorescent cells show the histochemical characteristics for a primary catecholamine, in this organ no adrenaline is to be found chemically There is also evidence from the histochemical data that some of the cells seen in the other animals may contain a primary catecholamine With the use of bichromate staining Euler (1934) observed chromaffin cells in the muscle septa of the cat prostate These chromaffin cells seem to be identical with the small branching cells exhibiting an intense fluorescence typical of catecholamines seen in the present investigation

It is likely that these "chromaffin cells often found in prominent clusters, especially in the organs of the macaque are in cases responsible for a considerable part of the catecholamine content present in the organs in question (*cf* Chapter V)

The peripheral adrenergic nerve cell bodies earlier mentioned are of various appearance In the guinea pig and the rat they are rather uniform in size and exhibit a green fluorescence of varying intensity In the dog and the cat they vary considerably in size, some being as large as the cells in the inferior mesenteric ganglion, but many being quite small In these specimens the small cells generally exhibit a much more intense fluorescence than the bigger ones In the macaque small, rather intense, fluorescent nerve cell bodies predominate *Concerning the macaque, which has a hypogastric ganglion like man it should be noted that this ganglion consists of large non fluorescent cells and only very few fluorescent neurons The peripheral adrenergic nerve cells are located chiefly in the walls of the seminal vesicle and the prostates*

Around some of the peripheral nerve cells are seen adrenergic terminals,

the incidence varying fom species to species No certain reduction in amount of these terminals is seen following hypogastric denervation and lumbosacral sympathectomy (cat), indicating that they emanate from cell bodies located within the peripheral cellgroups

The results presented in this chapter confirm the evidence obtained in the previous chapters that the adrenergic innervation of the vas deferens and the accessory male glands derives from short adrenergic neurons, located in close vicinity to the effector cells Thus adrenergic neurons having their cell bodies in the inferior mesenteric ganglion do not in any appreciable de gree take part in the adrenergic innervation of the vas deferens and the accessory male glands In the macaque the hypogastric ganglion is not the locus of adrenergic nerve cells innervating the accessory male organs These cells are located closer to the effector organs, mainly in their walls which may imply that this pattern is also present in other primates including man

The dense distribution of adrenergic terminals in most of the accessory male organs (in their smooth muscle compartments) corresponds well with the high noradrenaline content found in these organs (Chapter V) It should, however, be considered that there are species variations in the concentration of noradrenaline in the organs Besides differences in the relative amount of non innervated tissue compartments, these variations could be due to differences in density of the adrenergic terminals Thus in the rabbit- and the cat vas deferens there are wider meshes in the adrenergic network than in the rat and the guinea pig vas deferens, which may explain why the vas deferens of the rabbit and the cat has a lower content of noradrenaline than the vas deferens of the rat and the guinea pig (*cf* Chapter V) In the cat there is also a sparser adrenergic innervation of the funicular parts of the vas deferens than in the pelvic parts The difference in noradrenaline content between the vas deferens of the guinea pig and the rat (*cf* Chapter V) could be due to the presence of preterminal varicose fibers, obviously storing high amounts of noradrenaline, seen in the nerve bundles outside the vas deferens of the former species The very high noradrenaline content of the macaque vas deferens could be partly due to noradrenaline stored in chromaffin cells Finally it can not be excluded that there are species variations in the content of noradrenaline of the nerve terminals, which thus may cause species differences in noradrenaline content of the internal male genital organs

A diverging innervation pattern was found in the dog vas deferens A distribution of adrenergic fibers similar to the other animals occurred only in the inner longitudinal layer, whereas the other muscle layers contained only a scarse amount of fluorescent fibers This is in good accordance with the rather low content of noradrenaline in the dogs vas deferens (Chapter

V) However, silver impregnation revealed in the thick circular layer a rich plexus of nerve fibers, which then presumably is cholinergic It is of interest to note in this connection that the fox vas deferens has the same low nor adrenaline content as that of the dog

Recently the presence of adrenergic nerve cells in the pelvic plexus of the rat has been confirmed by Norberg and Hamberger (1964) in referring to Falck Owman and Sjöstrand They also present some photomicrographs from the " prostatic gland and the prostatic vesicle (cf Chapter II page 13 in this report) as examples of adrenergic ground plexa Their results from the vas deferens of the rat is discussed in chapter VII

CHAPTER VII

GENERAL DISCUSSION

From the experimental chapters it is concluded that the mammalian vas deferens and the accessory male genital glands, with some few exceptions, possess a very dense adrenergic innervation of their muscular layers This has been shown by estimation of the organ content of noradrenaline as well as by histochemical visualization of the adrenergic terminals

The adrenergic neurons constituting this dense innervation are short, i e their cell bodies are located close to the target organs This has been revealed by experiments with ganglionic blocking agents on the isolated hypogastric nerve vas deferens preparation the persistence of the tissue noradrenaline after sympathetic denervation and the direct demonstration of the adrenergic neurons innervating these organs

In addition to the neuronal store of catecholamines some of these organs, in certain species, contain special cells storing high amounts of catechol amines Probably most of them contain adrenaline, as indicated from histochemical data and the adrenaline levels in the organs

Exact details on the adrenergic innervation of the internal male organs i e the proportion of smooth muscle cells receiving varicose terminals, the number of synaptic varicosities on each muscle cell the degree of con vergence of the individual postganglionic neurons on each individual or group of muscle cells and the number of smooth muscle cells supplied by one nerve cell, are still lacking Burnstock and Holman (1961) (and later Kuri yama 1963) found a high degree of convergence of the hypogastric axons on the smooth muscle cells of the guinea pig vas deferens It should, however, be kept in mind that in their original report, the hypogastric nerve was regarded as a postganglionic nerve to the vas deferens Thus the convergence they found could partly take place in the peripheral synaptic relay demon strated by the present investigator (Sjostrand 1962 a, b) as pointed out by Merrilees Burnstock and Holman (1963) There is, however, certainly also a marked degree of convergence of the postsynaptic neurons on the effector compartments of the target organ due to the construction of the autonomic ground plexus (*cf* Hillarp 1959 and Falck 1962) Richardson (1962) in his electron microscopical investigation of the rat vas deferens concluded that every smooth muscle fiber came in close contact with one "synaptic

ending' On the other hand Merrilees, Burnstock and Holman (1963) considered it unlikely that every muscle cell in the guinea pig vas deferens came in close contact with an axon From the data presented in this investigation it seems likely that most—if not all—of the muscle cells of these species come in close contact with one or more adrenergic varicose terminals Similar conclusions were also drawn by Falck (1962) using the guinea pig vas deferens, and by Norberg and Hamberger (1964) using the rat vas deferens The present investigation indicates a similar distribution of adrenergic terminals in other mammals, the dog and probably fox being exceptions The smooth muscle compartments of the accessory male glands investigated have a distribution of adrenergic terminals of similar or somewhat lower density than the vas deferens

Since there is reason to believe that, in these smooth muscles, the excitation can spread electrotonically from one muscle cell to neighbouring cells (*cf* Prosser 1962, Burnstock, Holman and Prosser 1963, Merrilees, Burnstock and Holman 1963, Burnstock and Holman 1964), it may be of rather little physiological significance if each or only every second or third muscle cell is innervated It is, however, probably of importance, that by means of their dense innervation, these smooth muscular organs may respond rapidly and efficiently to a central neuronal discharge, conducted by the proportionally few preganglionic fibers in the hypogastric nerve (see Chapter I) to the rather numerous peripheral neurons

The vas deferens and the accessory male glands contract during the emission period, which in most species is rather short With their—generally—strong smooth muscle coats, abundantly supplied with nerve terminals the accessory male organs seem well adapted for a rather brief and forceful ejection In this respect the low noradrenaline content and sparse adrenergic innervation of the bulbourethral glands may be indicative, since in these glands the motor function is mainly performed by striated muscle

Further the smooth muscles of the internal genital organs seem also to have properties in common with typical smooth muscles of the multiunit group of Bozler (*cf* Bozler 1948) Thus they generally show no prominent motility when mounted in an organ bath, and in the case of the guinea pig vas deferens almost no such activity (Hukovic 1961, Burnstock and Holman 1961) The guinea pig vas deferens is very insensitive to smooth muscle stimulants (Sjostrand 1961) but responds efficiently to nerve stimulation (*cf* Burnstock and Holman 1961) *In vivo* these organs are probably quiescent unless they are activated by a neuronal discharge There is also no permanent tonic discharge to these organs as judged from the findings of Adrian, Bronk and Phillips (1931) (see Chapter I)

Some glands, such as the seminal vesicle of the boar and the ventral parts of the prostates of the rat and the dog have a comparatively sparse supply of smooth muscles and, in addition also a rather sparse adrenergic innervation *around their prominent secretory compartments*

These glands can probably not deliver their preformed contents or—as is generally the case in emission—parts of it at the same rate as organs apparently built for a brief ejection of their contents like the sacculous seminal vesicle of the rabbit with its thick smooth muscle coat having a dense adrenergic innervation In this connection it may be of interest to compare the copulation times of the rabbit and the boar In the rabbit the entire copulation may be finished within 5 seconds while the boar copulates in about half an hour This long copulation time in the boar may at least partly be due to a slow delivery of the rather voluminous secretion present in its large, tubulo-alveolar seminal vesicles (*cf* Mann and Lutwak Mann 1951 and chapter II), having rather few smooth muscle cells surrounding the acini and a rather low content of the adrenergic transmitter

Innervation of the target organs by short adrenergic neurons seems to be a rather unique pattern in mammals where the adrenergic cell bodies usually are located in the sympathetic chain or the prevertebral ganglia Thus the scheme of adrenergic innervation in the internal genital organs resembles that seen in the parasympathetic nervous system Recently a similar arrangement has been described for the trigonum of the bladder in cat (Hamberger and Norberg 1965) About one third of the adrenergic innervation of the Fallopian tube of the rabbit seems also to derive from short adrenergic neurons (Brundin pers communication) In lower vertebrates there are also examples of this kind of sympathetic adrenergic innervation (Fahlen Falck and Rosengren 1965)

The functional significance—if any—of this peculiar pattern of adrenergic innervation can at present only be a matter of speculation It may be that a short preterminal distance of the axon permits a larger number of the terminal ramifications or a greater length of the terminals thus innervation by short adrenergic neurons of a target organ may possibly facilitate the formation of a dense adrenergic ground plexus in the target organ Further innervation of an effector organ by short adrenergic neurons makes it possible to achieve a dense adrenergic ground plexus in the organ without increasing the "extrinsic" innervation Finally the localization of the adrenergic ganglia close to the field of action may be of some advantage for the central nervous system in exerting restricted effects upon the internal male organs as in the case of emission Thus the effect of a sympathetic discharge to these organs may be directed with a greater precision of localiza

tion than those seen in other areas on sympathetic discharges, and may accordingly emulate the localized effects of parasympathetic discharges to the effector organs

The physiological significance of the "chromaffin" cells in the accessory male organs of the macaque and the prostates of the dog and the cat is at present obscure and needs further investigation It is not even known if these cells are innervated in the same manner as the chromaffin cells of the adrenal medulla or if they are not innervated, which some authors claim to be the case for the chromaffin cells of the paraganglia (*cf* West *et al* 1953)

During the recent years there has been discussion of the localization of the small amounts of adrenaline present in various organs as for example the heart and the salivary glands In view of this discussion it may be of interest to note that, in the present study, adrenaline in the accessory male genital organs has been found to correspond to the presence of "chromaffin" cells, displaying histochemical criteria of a high content of adrenaline Hitherto such cells have not been satisfactorily demonstrated in peripheral mammalian tissues This may be due to a very sparse distribution of "chromaffin" cells in most tissues, why they may have escaped detection This assumption seems to be justified by the very low levels of adrenaline present in most organs Consequently the present results supports the opinion of Euler (*cf* Euler 1956, 1961, 1963) that adrenaline in mammalian tissues is mainly stored in chromaffin cells

Finally it should be stated that the adrenergic nerve plexus in the muscular walls of the internal male organs described in this report does not exclude an additional cholinergic plexus in the muscularis But the possible existence of such a plexus has hitherto little experimental support To judge from the data given by Risely and Skrepetos (1964), there seems to be no correlation between cholinesterase positive structures in the rat vas deferens and the adrenergic fibers The cholinesterase activity probably outlines a system of cholinergic neurons, which run in the muscle coat of the vas deferens to the secretory epithelium In the vas deferens of the dog and presumably the fox there is however, probably a cholinergic innervation of the muscles of the vas deferens This is one more example of the puzzling species differences concerning autonomic innervation of homologous organs which occur rather frequently in the animal kingdom

In this respect it should also be pointed out that no evidence has been obtained in this investigation supporting the theory of Burn and Rand (Burn 1960, Burn and Rand 1960) that the adrenergic transmitter in sympathetic neurons is released by some kind of "cholinergic mechanism" in these neurons On the contrary the present finding of short adrenergic neurons (and chro-

maffin cells) may well constitute a conceivable explanation of at least many of the experimental data obtained from different preparations, apparently supporting this theory if it is assumed that some short adrenergic neurons (or chromaffin cells) are present in most organs receiving an adrenergic innervation

SUMMARY

The adrenergic innervation of the vas deferens and the accessory male glands of some mammals and the presence of adrenaline and 'chromaffin' cells in these organs were investigated by the following methods

1 The effect of ganglionic blocking agents was studied on the isolated guinea pig vas deferens hypogastric nerve preparation

2 The catecholamine content of the vas deferens and the accessory male glands of hedgehog, macaque, rabbit, rat, mouse, guinea pig, dog, fox, cat, boar, bull, and ram and the vas deferens of man was estimated fluorimetrically Estimation of catecholamines after hypogastric denervation was performed on rabbit, macaque, rat, guinea pig, dog, cat and ram, after pelvic denervation on dog and after lumbosacral sympathectomy on cat

3 The cellular localization of the catecholamines in the internal accessory male genital organs of rabbit, rat, guinea pig, dog, cat and macaque was studied with a fluorescence microscopic method The effect of hypogastric denervation was investigated on rabbit, rat, guinea pig, dog and cat and the effect lumbosacral sympathectomy on cat

The following results were obtained

1 The motor response of the guinea pig vas deferens to hypogastric nerve stimulation was blocked by ganglionic blocking agents Decentralization of the inferior mesenteric ganglion almost abolished the response to hypogastric nerve stimulation but 'normal' contractions were elicited if the stimulation electrodes were placed close to the vas deferens These responses could not be obliterated by ganglionic blockers

2 Most of the accessory male organs and above all the vas deferens contained exceptionally high concentrations of noradrenaline There seemed to be a gross correlation between noradrenaline content and amount of smooth muscle in the organs, thus organs with a high proportion of glandular parenchyma had a lower content than organs possessing strong muscular coats The vas deferens of dog and fox had an exceptionally low noradrenaline content when compared with the other mammals (and also with the cock and the tortoise) The bulbo-urethral glands had a low noradrenaline content

No significant reduction in organ content of noradrenaline was seen after hypogastric denervation Pelvic denervation in the dog caused no obvious decrease in noradrenaline content of the vas deferens After lumbosacral

denervation there was a moderate decrease of noradrenaline in the cat vas deferens

The internal male genital organs of the macaque contained besides noradrenaline also remarkably large amounts of adrenaline Adrenaline could also be found in the prostate of the dog

3 The high content of noradrenaline corresponded with a very dense distribution of adrenergic terminals in the smooth muscle of the organs Secretory cells seemed not to have an adrenergic innervation The vas deferens of dog had a sparse adrenergic innervation but a dense network of nonadrenergic nerves

The adrenergic terminals of the internal male genital organs persisted after hypogastric denervation After resection of the lumbosacral sympathetic chain there was a marked reduction in vascular innervation of the vas deferens and the prostate of cat but no visible decrease in amount of adrenergic terminals in the muscular coats

The adrenergic fibers of the internal male genital organs were found to originate from adrenergic cell bodies located close to or within the walls of these organs

In the vas deferens the seminal vesicle and the prostate of the macaque and in the prostates of dog and cat small polymorf cells with short processes containing high amounts of catecholamines were present Most of these "chromaffin" cells in macaque and dog seemed to contain adrenaline, those of the cat prostate showed histochemical characteristics of a primary catecholamine

From the results it is concluded that

1 The vas deferens and the accessory male genital glands of the examined species—in contrast to the general pattern of adrenergic innervation in mammals—are innervated by short adrenergic neurons and have an exceptionally rich adrenergic innervation

2 Adrenaline in the internal male genital organs is mainly stored in "chromaffin" cells

It is further suggested that the rich adrenergic innervation of the vas deferens and the accessory male genital glands is of importance for the emission

It is also suggested that the innervation of these organs by short adrenergic neurons may be of importance for the emission

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GÖTEBORG
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INDEX

FROM THE DEPARTMENT OF CLINICAL PHYSIOLOGY, UNIVERSITY OF GÖTEBORG, SWEDEN

ON THE COMPONENTS OF Kr^{85} CLEARANCE CURVES FROM THE BRAIN OF THE DOG

by

EGIL HÄGGENDAL, NILS JOHAN NILSSON and BENGT NORBÄCK

ABSTRACT

EGIL HÄGGENDAL, NILS JOHAN NILSSON and BENGT NORBÄCK. *On the components of Kr^{85} clearance curves from the brain of the dog.* Acta physiol. Scand. 1965. *66*. Suppl. 258. 5—25. — Clearance curves from the brain in dogs were studied after short intra-arterial injections of Kr^{85}. Multiexponential curves consisting of two main components were received both by recording γ and β radiation. The slopes of these two components corresponded to each other in the two types of curves. Local micro-injections of Kr^{85} in the cortical grey matter resulted in mono-exponential clearance curves with the same slope as the fast phase of the composite γ or β curve, whereas curves from micro-injections in the subcortical white matter had a slope corresponding to the slow phase of the composite curves. A third very slow phase of the composite γ curve was identified as due to flow in extracerebral tissue. In addition, an initial rapid phase was recorded in most curves after intra-arterial injections and was assumed to be due to activity in the arterial blood. This phase was not present in clearance curves from blood drawn from the superior sagittal sinus, whereas the two main components representing blood flow in grey and white matter were identified. Some difficulties in the analysis of the composite clearance curves, especially concerning the slow component, are discussed.

INTRODUCTION

In 1961 a new method for determining regional blood flow of the cerebral cortex was published by LASSEN and INGVAR. The method, a modification of that described by KETY *et al.* (1955), is based upon the external counting of the β radiation from the exposed surface of the brain following intra-arterial injections of an inert radioactive gas. KETY's original technique measured the incorporation rate in the brain and required decapitation of the experimental animal. This new method with many of its theoretical considerations was described more fully by INGVAR and LASSEN (1962). A similar method for determining cerebral blood flow was introduced by LASSEN *et al.* in 1963 by making use of the γ radiation of radioactive krypton (Kr^{85}) for extracranial recording. These methods, primarily described for blood flow measurements of the brain, have since then also been used for determination of blood flow in other organs as originally suggested by the authors. HARPER and his coworkers have studied cerebral blood flow with the method of LASSEN and INGVAR in its original form (HARPER, GLASS and GLOVER 1961; HARPER and BELL 1963) as well as with Xe^{133} instead of Kr^{85} as an indicator (HARPER *et al.* 1964; HARPER 1964). Extracranial recording of the γ radiation after intra-arterial injections of Kr^{85} has been applied both in animal experiments (HÄGGENDAL and JOHANSSON 1965) and in clinical studies (INGVAR *et al.* 1964; HÄGGENDAL *et al.* 1965).

The greatest advantage of these methods is that they involve no trauma to the brain, require no blood sampling and are readily repeatable. Their interpretation is complicated, however, by the fact that the desaturation curves recorded from either γ- or β radiations do not represent single exponential functions. They are ordinarily regarded as being composed of one fast and one slow phase. INGVAR and LASSEN (1962) stated that the cerebral cortex is a non-homogeneously perfused tissue which yields the two separate components of the β clearance curve. In the case of the γ curve the two components are generally regarded as representing flow in two different types of brain tissue. Thus, if it were possible to establish which tissues these are, it would be possible to obtain differentiated flow values from the composite organ. LASSEN *et al.* (1963) and HOEDT-RASMUSSEN (1964) assume that the fast component represents blood flow in the grey matter of

the brain and the slow component flow in the white matter but no experimental evidence for this assumption has been given so far

The purpose of the present study was to analyze the type of composite curve obtained after intra arterial injections of Kr^{85} To this end desaturation curves of β radiation from the cortical surface and of γ radiation from the greater part of the brain have been recorded simultaneously or in immediate succession Also local micro injections into different regions have been used to identify the single components of the composite curves A comparison has been made with the curve obtained from blood from the superior sagittal sinus In addition the difficulties in the analysis of composite exponential curves will be discussed

METHODS

The study was performed in 25 unselected mongrel dogs with body weights from 6—15 kg (plus one dog of 35 kg) under pentobarbital anaesthesia The animals were artificially ventilated through an endotracheal tube and most of them curarized Arterial blood pressure was observed continuously and recorded during the measurements of cerebral blood flow (CBF) Analyses of arterial carbon dioxide tension and oxygen saturation were done at intervals of about 2 hours Clotting was prevented by intravenous administration of heparin Successful experiments lasted 8—10 hours For further details concerning this general procedure the reader is referred to the paper of HÄGGENDAL and JOHANSSON (1965) By changing the ventilation and the composition of the inhaled gas the animal could be studied in very different respiratory states which could be kept constant for long periods To check the constancy of the arterial carbon dioxide tension 27 pairs of consecutive analyses in unchanged conditions were studied comprising partial pressures ranging from 15 to 64 mm Hg The mean change from the first to the second analysis was 0.67 ± 0.53 mm Hg which is not statistically significant ($P > 0.20$) and the greatest deviation encountered once was 5 mm Hg

Determinations of CBF were performed according to the principles of the two methods of INGVAR and LASSEN (1962) and LASSEN *et al* (1963) A thin polyethylene catheter (PE 110 or 160) was introduced into the intact vertebral artery as described by HÄGGENDAL and JOHANSSON (1965) In some of the experiments a catheter was also inserted via the cut lingual artery into the external carotid artery so that the tip lay just cranial to the carotid bifurcation The external carotid artery was ligated so as to minimize extracerebral blood flow The catheters were filled with heparin saline solution and small amounts of this solution were given at intervals to flush the catheters

After the catheterization of the vertebral artery a small craniotomy was performed in the parieto temporal region with a trepan (18 mm diameter) Bleeding from the bone was controlled with bone wax In most of the dogs the dura was removed before the measurements of flow were started When local injections were to be done two craniotomies were performed with the second about 10—15 mm frontal to the other

The γ activity was measured with a scintillation detector (2 in NaI crystal) coupled to a ratemeter (Nukleonik AB Goteborg) with a time constant of either 0 3 or 1 sec The detector was placed over the skull so that the major part of the homolateral hemisphere was seen by the crystal The influence of extracerebral activity was reduced as much as possible by lead collimation Contamination of the air in the room with Kr^{85} was avoided by carrying off the animal s expired air through a tube to the outer air

The β activity was measured with a Geiger Müller end window tube (Philips no 18504) The tube was connected to another ratemeter of the same type as was used for the scintillation detector and the same time constant was used The tube was placed about 1 mm over the cortical surface and was shielded from the surrounding bone by a lead cuff The cortical area recorded from amounted to about 65 mm²

The radioactive isotope Kr^{85} (Radiochemical Centre Amersham England) was dissolved in saline For the intra arterial injections the volume of the Kr^{85} solution was about 1—3 ml and the injections usually lasted 3 seconds

Local injections of the radioactive solution were performed by means of a small syringe (no 701 Hamilton Ltd Whittier California) with a thin needle (0 4 mm external diameter) in a manner analogous to that used for similar injections in muscle (LASSEN LINDBJERG and MUNCK 1964) The syringe was fixed to a stand so that it could be placed into the desired position in front of the frontal trepan hole The needle was then brought fairly superficially through the cortical or subcortical matter until it was in position below the GM tube which was placed over the other trepan hole No significant increase of the background activity was measured by the GM tube with the needle and syringe in this position The locally injected volumes were 1—5 μl The needle was kept *in situ* during the measurement of flow in order to prevent leakage of the injected fluid along the injection tract Many of these injections were performed with dyed solutions of Kr^{85} in order to enable as exact a post mortem localization of the injection site as possible The dyes used were Congo Red Trypan Blue and Kiton Fast Green in approximately 5% solutions After the local injections the animal was sacrificed, the brain cut into slices and the dyed spots localized

The activity after both intra arterial and local administration of the isotope

was recorded on potentiometer writers of different types Micrograph BD 3 (Kipp) capable of high paper speeds and a Beckman Laboratory Potentiometric Recorder to enable logarithmic recordings to be made

The injections gave maximal counting rates of from 5000—50000 cpm When correcting for the coincidence loss the measured value of 93 μsec for the dead time of the GM tube was used

In evaluating the recorded curves we have used the ordinary method regarding each composite curve as representing the sum of several exponential functions Thus the process must be followed long enough for the last part of the curve to give a straight line plotted semilogarithmically This straight line is then extrapolated backwards to zero time its linear values subtracted from those of the original curve to give a curve of the difference values which is treated in the same way as the original curve and this process of subtraction is repeated until the original curve has been completely resolved into a number of straight lines By this procedure it is possible in theory to determine the number of individual exponential functions constituting the curve as well as their respective slope constants Also an inspection of the semilogarithmic curve would reveal if observations have been carried out long enough If not its final portion would not become straight In practice however when applying this technique we have confirmed the impression of others (BRISCOE and COURNAND 1959 BERGNER 1963) that the method has very definite limitations To a great extent these are due to the statistical variations in the measured activity Because the ratemeter curve is always more or less wide and irregular the amplitude to be plotted on the semilogarithmic paper cannot be very precisely defined The influence of this uncertainty will be more pronounced as the activity decreases Thus it is often possible to draw an acceptably fitting straight line through the last part of the curve even if it has been recorded for a shorter time than theoretically necessary An example is given in Fig 1 in which a curve is analyzed in two alternative ways either (dashed lines) being resolved into three components using the part of the curve recorded after more than 30 minutes or (drawn out lines) into only two components neglecting all points after 17 minutes i e regarding the curve as not having been recorded for longer a time In actual practice therefore it is normally not possible to deduce from the curve how many components it contains An important point is that the uncertainty increases towards the end of the curve Thus in the example given the component with the steepest slope comes out practically identical in both cases The values for the slope of the second steepest component on the other hand are clearly different although both are of the same order of magnitude For reasons given later we regard these components as representing blood

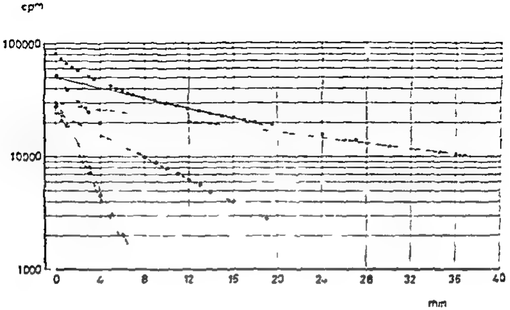


Fig. 1. Example of the uncertainty in curve interpretation. A γ curve following intra-arterial injection towards the end of an experiment when extracerebral activity had become high is resolved in two ways: 1) into 3 components (dashed lines) with half time values 1 [illegible] 6.0 and 23 [illegible] min. 2) into 2 components (drawn out lines) with half time values 1.[illegible] and [illegible] min. The latter resolution would have ensued if the curve had not been observed for more than 15 minutes. Note the great difference in slope of the middle component (white matter) and the identity of the slope values for the rapid component (grey matter).

flow in grey and white matter of the brain respectively and the very slowly falling component to represent flow in extracerebral tissues. The error in the determination of flow rate is therefore considerably smaller for grey than for white matter, especially since the difference in flow rate between these two is always large, usually about five fold. Thus in most cases an observation time of 10—15 minutes will be satisfactory for the determination of flow rate in the grey matter, a circumstance which is of practical importance when repeated determinations are to be performed in rapid succession without change in the state of the animal. For these reasons we have generally placed more emphasis on conclusions drawn from the flow rate determinations from grey than from white matter.

In a series of double determinations the deviation within each of 23 pairs of determinations of the rapid phase (grey matter) in which flows ranged from 36 to 220 ml/(100 g · min) exceeded 10 % in only 4 cases. On the other hand, measurements of the slow phase (white matter), where the range of

flow was from 6 to 45 ml/(100 g · min) revealed a 10% deviation in 8 out of 17 later injections. Of these latter cases, however, only 1 diverged more than 5 ml/(100 g · min).

RESULTS

As mentioned in the introduction, γ clearance curves of radioactive inert gases from the brain following intra-arterial injections, detected externally, are composite curves. They have generally been resolved into two components with rather different slopes which have been tentatively taken to represent flow in the two main macroscopic structures of the brain, grey and white matter. In order to analyze this situation further we have undertaken several types of experiments.

Comparison of γ and β activity after intra-arterial injection

The afore-mentioned hypothesis regards the two brain substances as relatively homogeneous with respect to blood flow. Therefore, if it were possible to record the activity from either white or grey matter exclusively, it would seem plausible to expect the clearance curve to proceed as one single exponential function. Such a possibility might be afforded by recording the β activity from the exposed brain surface, assuming the thickness of the cerebral cortex to exceed the range of the β emission. In making such recordings, however, we have verified the findings of INGVAR and LASSEN (1962) that the curves are in fact multiexponential (*cf.* Fig. 2 b).

This might be explained by assuming either the existence of different blood flows in different parts of the cortex (INGVAR and LASSEN 1962) or an admixture of activity from the underlying white matter. To investigate this problem we have made 44 measurements in 17 dogs with both γ and β desaturation curves after intra-arterial injections of Kr^{85}, either simultaneously or in immediate succession. As illustrated in Fig. 2 both types of curves can be resolved into two, or more often, three components. The slopes agree quite well with each other within each pair of curves, the agreement being better for the more rapidly declining components, as discussed in the Methods section. Of these phases the fastest one, which was not always present, will be discussed later. The two others have slopes of the same order of magnitude as the fast and slow phases ordinarily obtained from composite cerebral desaturation γ curves. Therefore we have calculated flow from these slopes, assuming them to represent grey and white matter, respectively.

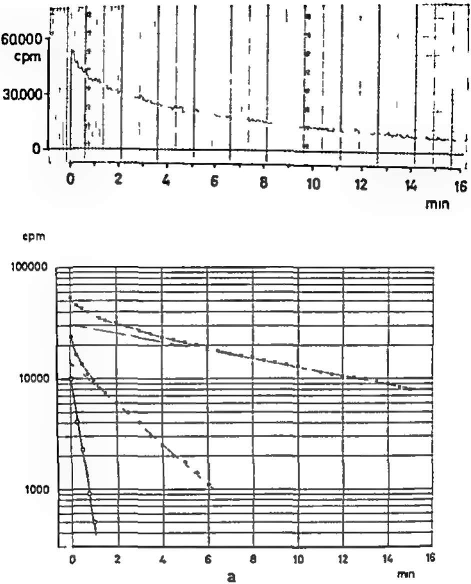
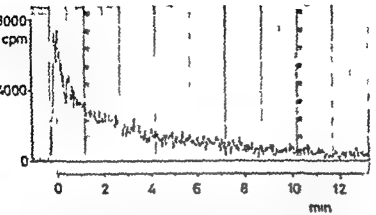
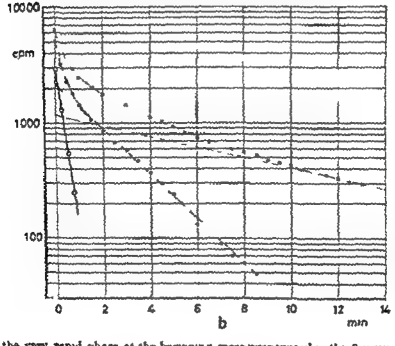


Fig 2 *a* and *b* Corresponding pair of original curves and semilogarithmic replottings of γ (a) and β (b) radiation

i e using the corresponding partition coefficients of 0 95 and 1 30 (LASSEN *et al* 1963) for the calculations By varying the arterial carbon dioxide tension in animals with artificial respiration it was possible to obtain flows varying between about 35 and 220 ml/(100 g min) for the grey substance and between 6 and 45 ml/(100 g min) for the white The correlation between the pairs of γ and β curves is illustrated in Fig 3 The good agreement indicates that the respective curve components originate in the same type of tissue This would





b

Note the very rapid phase at the beginning more pronounced in the β-curve The half time values for the three components are in the γ curve 0 2" 1 65 and " 85 min. in the β curve 0 0 1 80 and 6 65 min.

verify the interpretation of the fast γ phase as emanating from the grey matter but would on the other hand indicate that the slow component of the β curve is derived from the white matter Otherwise it would be necessary to postulate the existence of one part of the cortex having a blood flow the same as that of white matter

Although the β activity accounts for more than 99% of the total emission

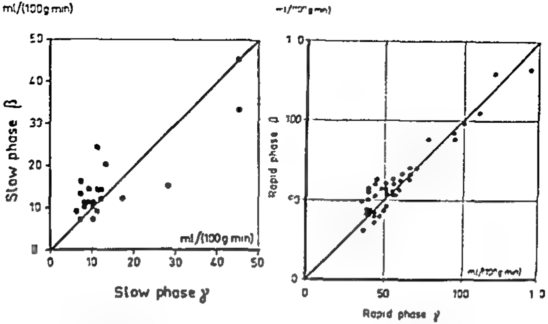


Fig 3 Comparison of the two main components of the composite γ and β curves The flow values are calculated for the rapid and slow phases using the partition coefficients for grey and white matter respectively The line of identity is shown

from Kr[85] and the sensitivity of the GM tube towards γ activity is low one must consider the fact that the tube may receive γ activity from the whole hemisphere i e a volume about 100 times greater than that from which β activity can reach it

However interposing an aluminium sheet 1 mm thick, enough to absorb the β radiation but without noticeable influence on the γ activity reduced the total activity measured to 1 or 2% a value which is obviously smaller than the relative contribution of the slow phase (cf Fig 2 b) On the other hand after a large injection it was possible by increasing the gain on the amplifier to identify this very low activity as an ordinary γ curve composed of the usual two components

In view of this the most probable source of the slow phase of the GM curve would be β radiation from the white matter The plausibility of such a conclusion is supported by the fact that actual measurements have shown the thickness of the cortex in the dogs used in these experiments to be about 2 mm whereas the range of the β particles from Kr[85] in water according to the data obtained from the Radiochemical Centre can be calculated to be around [illegible] mm In one dog weighing 35 kg whose cortex was 3 mm thick the β curve from the cortical surface was monoexponential

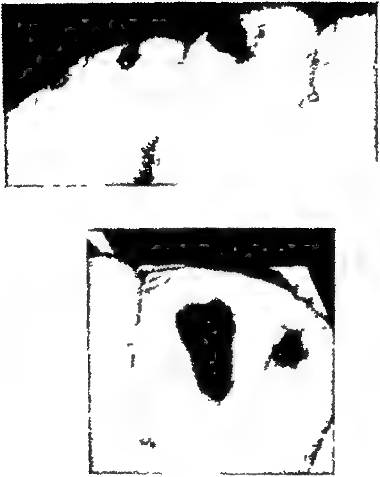
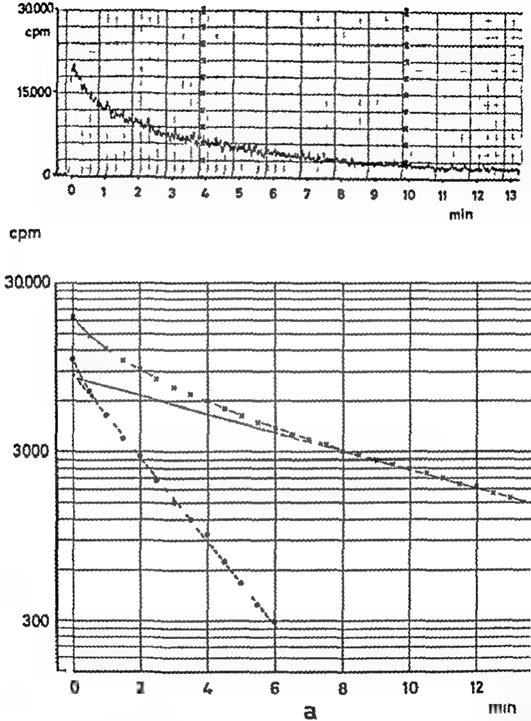
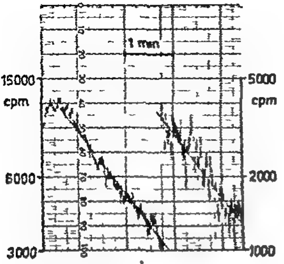


Fig 4 Brain sections — from the same animal as in Fig 3 — giving the localization of micro injections of the accompanying dyes



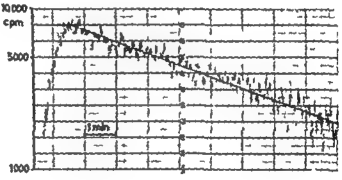
a

Fig 5a Example of corresponding composite curve and curves from local micro injections a) γ curve original and semilogarithmically replotted Half time values of the two components 1 *0 and 5 40 min



b

Fig 5 b Local injection in grey matter (green in *Fig 4*) Logarithmic recording amplification increased 10 fold in the middle of the curve Half time 1 10 min



c

Fig 5 c Local injection in white matter (red in Fig 4) Logarithmic recording *Half time 4 0* min

The value of 1 0 for the partition coefficient of krypton between muscle and blood has been used for the calculation of flow (*cf* HANSEN *et al* 1950) We are not able to make the corresponding calculation for connective tissue, as its partition coefficient has not been determined

On another occasion a γ curve was recorded with the detector over the neck after an injection in the vertebral artery A practically purely monoexponential curve resulted presumably mainly from neck musculature with a half time of 12 ▪ min corresponding to a flow of between 5 and 6 ml/(100 g min)

We regard these findings as evidence in favour of our view that the very slow component found after long observation times which is discussed in the chapter on Methods and illustrated in Fig 1 is really representative of flow in extracerebral tissues rather than in extremely slowly perfused parts of the brain

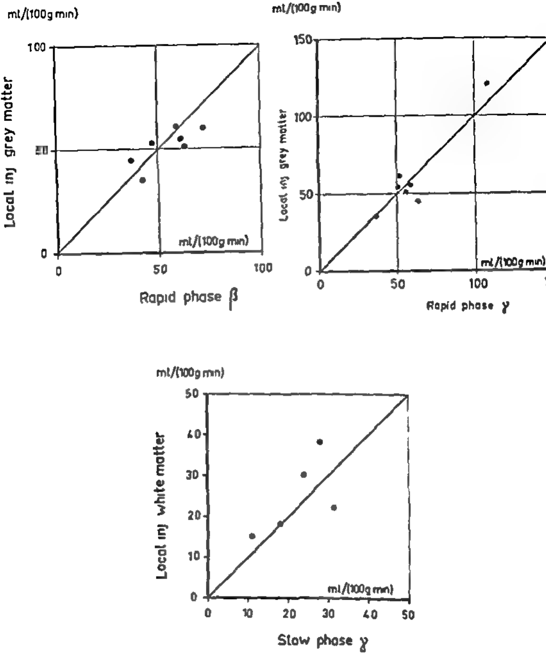


Fig [illegible] Comparison of flow values from curves after local injections and from corresponding composite γ or β curves Injections in grey matter compared with the rapid main phase in the γ and β curve injections in white matter compared with the slow phase in the γ curve Each point represents the mean value of 1—4 local injections made with the animal in the same state compared each time with one and the same composite curve The line of identity is shown

It may be mentioned that in some instances injections in the vertebral artery did not give rise to any measurable β activity in the anterior trepan hole though activity was always found in the posterior hole as it was in both holes after injections in the carotid artery This is in agreement with the opinion that the vertebral artery delivers blood mainly to the posterior part of the brain

Relation between cerebral flow rates

Without exception the flow rate calculated for the grey matter with the present method is several times greater than that for white matter In 83 determinations in our series the average value for the quotient between grey and white matter flows was found to be 5 2 In these determinations the flow in the grey matter varied between 36 and 220 and that in the white matter between 6 and 45 ml/(100 g min) There was no systematic difference between the quotients at low and at high flow rates Only 7 quotient values were outside the limits 3 and 10 This is well in accordance with the findings of other investigators (SOKOLOFF 1961 see also GLEICHMANN *et al* 1962) Considering the limited precision in the determinations of the flow in white matter we have however not found it appropriate to analyze these values further

Initial component of the clearance curve

As has been mentioned earlier a component with a much more rapid fall than that ordinarily attributed to the grey matter can usually be found at the beginning of the composite curves Because of its steepness its influence is noticed only for ½—1 minute Its half time is usually around 0 10 sometimes up towards 0 20 minutes We have considered the possibility that this could be the effect of an injection artefact (*cf* NILSSON 1960 p 20 for a description) the temporary augmentation of blood flow brought about through vasodilatation by substances liberated from erythrocytes in a minute hemolysis following a forceful intra arterial injection The phenomenon has been observed in the human brain by NILSSON (1957) This explanation seems improbable however in the first instance because our injections have been made much slower than those generally causing the artefact Furthermore when clearance curves were recorded from blood drawn from the superior sagittal sinus as described below this initial component could not be observed and

lastly it might perhaps be expected that an actual change in blood flow would not give such a good fit when falsely interpreted as an additional exponential function

It would seem more plausible to regard this extra activity as emanating from the indicator in the arterial blood where its concentration is naturally much higher than in the tissues and from which its clearance must be very rapid The initial part of the curve is somewhat reminiscent of the high and narrow peak seen at the beginning of the clearance curves from patients with intracranial arterio-venous fistulas (HAGGENDAL *et al* 1965) which must be explained in this way

This rapid component was more pronounced in the β than in the γ curves (*cf* Fig 2) and most after short rapid injections It was almost impossible to distinguish when the blood flow was very high and it was never seen after local injections of the indicator The possibility of a rapid diffusion of krypton from the superficial tissue layer into the air has been considered but in an experiment when a large amount of indicator solution was injected intra arterially immediately after the death of the animal the ensuing β activity could be observed to remain practically constant

In two animals 5 curves were recorded after intra arterial injection of non-diffusible radioactive isotopes twice with I^{131} labelled polyvinyl pyrrolidon (from the Radiochemical Centre Amersham) and three times with erythrocytes labelled with Cr^{51} in the manner usually used for blood volume determination In all cases the half times for the clearance of these blood borne indicators were between 4 and 7 seconds

Activity in sinus blood

For a long time there has been some discussion about the origin of the blood in the superior sagittal sinus It has been suggested that this blood would derive more or less exclusively from the cerebral cortex (*cf* GLEICHMANN *et al* 1962) It would seem that the method used by us might offer a possibility to test this conception Therefore in some dogs the sinus was punctured with a sternal needle and blood drawn continuously by a motor driven syringe through a counter tube (F 10 20th Century Electronics Ltd)

The curves recorded were composed of two phases the slopes of which were similar to those of the main components of the corresponding γ curves as shown in the table below

	Flow ml/(100 g min)			
	grey matter		white matter	
	γ curve	sinus blood	γ curve	sinus blood
Dog I				
Curve 1	51	47	9	13
Curve 2	29	23	[illegible]	9
Curve 3	63	86	17	15
Dog II				
Curve 1	40	43	9	10
Curve 2	148	132	20	21

From this it seems that the superior sagittal sinus receives blood from both grey and white matter

In these determinations blood was drawn at a rate of 1 3 ml/min In another dog the rate was varied from 1 5 to 8 ml/min with the animal in the same state without any significant change in the appearance of the curves

DISCUSSION

When the method of measuring blood flow by analyzing the clearance curves of β radiation from the cortical surface following intra arterial injections was introduced it was assumed that all the β radiation emanated from the cerebral cortex of the dogs The observation that the recorded curves consisted of two separate components therefore led to the conclusion that the cortex contained tissues with rather different blood flow values The results presented here indicate that the slow component of this curve may be derived from the immediate subcortical white matter

The initial slope method for the determination of blood flow (Ingvar and Lassen 1962) based on the cortical β curves demands that the initial concentration of the isotope is the same at the onset of clearance in all tissue recorded from If both white and grey matter i e tissues with different tissue blood partition coefficients for krypton contribute to the curve it will not be possible to achieve this identity of concentration Generally one might expect the participation of white matter to make the flow values which are regarded as representing grey matter too low However as pointed out in this study most β curves obtained after short intra arterial injections contain

an additional very rapid component. If this component is present also after the long infusions used in conjunction with the initial slope technique, it would tend to make the initial slope steeper than it would otherwise have been.

Concerning the question of the homogeneity of grey and white matter in respect of blood flow this study cannot give any definite answer. The evidence derived from it, however, is mainly against the existence of great differences within each substance. Thus the flow values from the γ curves, representing presumably the greater part of the hemisphere, correspond very well with the values from the locally limited β curves after arterial injection as well as with those from the very small local injections. Even the local injections in the superficial part of the medulla gave the same flow values as those from subcortical tissue. Injections made in the carotid artery gave the same flow values from γ curves as injections made into the vertebral artery, although the distribution of blood flow from these arteries was not always the same. This was shown by the fact that injections in the vertebral artery were sometimes not followed by any activity in the trepan hole over the anterior part of the brain, whereas carotid injections always provoked activity at both sites.

It must be stressed, however, that all our observations have been made on dogs under barbiturate anaesthesia, which is reported to abolish differences in blood flow in different parts of the cortex (SOKOLOFF 1961) and also to make the EEG pattern uniform (GLEICHMANN *et al.* 1962).

Since the cortex is only about 2 mm thick it may be impossible to analyze it for differences in blood flow between its superficial and deep parts with our micro injection technique. Also the question as to whether a monoexponential desaturation curve will be obtained when the cortex is thick enough to absorb with certainty all β radiation from underlying structures, has not yet been satisfactorily penetrated. Our single experience with a big dog speaks in favour of this assumption. Investigations of this problem in man are planned. Similarly an investigation of the homogeneity of flow by means of the γ radiation from local micro injections in the deeper parts of the white matter and in the basal ganglia and other deep grey structures is in a preliminary stage.

In this connection the problem may be raised as to how large is the tissue volume from which the locally injected isotope emits its activity. This question cannot be answered with certainty. The dye injected together with the indicator has been observed to spread 2—4 mm from the injection point, which is sometimes marked by a minute malacia. However, the spread of the dye cannot be claimed to be identical with that of the indicator. Since the dye is observed only after the killing of the animal, it has had a much longer time

to diffuse also it is not being removed by the blood as fast as the indicator is On the other hand the krypton gas will probably diffuse more rapidly than the dye When a local injection is given near the surface activity is recorded as soon as the injection is finished when given 6 mm or more under the surface it cannot be detected at all Intermediate injections however given at a depth of about 4 mm sometimes show a peculiar phenomenon which can be seen in Fig 6 c Immediately after the injection hardly any activity can be observed but in the course of the first ½—1 minute it builds up to a certain level from which it then disappears exponentially We have interpreted this initial rise as an expression of the diffusion of the indicator towards the GM tube whereas the central point of the injection is outside the range of the β radiation From the values given above a radial diffusion distance for the krypton of 1 or possibly 2 mm in white matter would be concluded.

After rapid intra arterial injections of indicator an initial very fast component is seen in the recorded curve which most likely is due to the activity of the injected isotope in the arterial blood It has presumably been observed by other investigators because it seems to be present not only in the brain but also in other tissues such as muscle and bone (own unpublished observations) The influence of this component on the clearance curve will be greater the higher the concentration of the isotope in the arterial blood is and the greater the volume of the arterial blood compared with the tissue For that reason the initial component is most pronounced after very short and rapid administration of the isotope into the artery and recording over the cortex which is more vascularized than the white matter Interpreted in this way it may permit estimation of the ratio of blood to tissue within the area recorded from

SUMMARY

Cerebral blood flow was studied in 25 dogs under pentobarbital anaesthesia by observing the tissue clearance of injected Kr^{85}

In one series of measurements the indicator was given in the carotid or vertebral artery and the time course of the γ emission detected by a scintillation crystal from the whole brain was compared with that of the β emission recorded by a Geiger Müller end window tube over the exposed brain surface By changing the arterial P_{CO_2} blood flow was varied over very wide limits Both γ and β curves could be resolved into two main components The slope of each one of the two components was always the same in γ and β curves recorded with the animal in the same state

In another series local micro injections of Kr^{85} were given in the cortex or the subcortical white matter and the β emission recorded from the surface. The exact site of each injection was established by post mortem examination of the brain with the aid of different dyes in the injected solutions. When the injections were made in grey or white matter the ensuing curve was always monoexponential with a slope corresponding to the fast or slow phase, respectively, of the composite γ or β curve from the animal in the same condition. When the injection was made on the borderline between grey and white matter it resulted in a composite curve consisting of the same two main components as the corresponding γ curve.

A very slow phase often encountered, especially towards the end of the experiments, was identified by local injections as representing flow in extra cerebral tissues.

A very rapid phase observed especially at the beginning of the β curves is described and attributed to activity in the arterial blood.

The curves of the activity in blood drawn from the superior sagittal sinus after an intra arterial injection can be resolved into two phases with slopes corresponding to those of grey and white matter.

It is concluded that the two main components of the composite cerebral desaturation curves after intra arterial injections represent flow in grey and white matter respectively and that this is true for the β curves recorded from the brain surface as well as for the γ curves from the whole hemisphere. No evidence of differences of blood flow within grey or white matter has been found.

Some difficulties in the interpretation of the composite curves are commented upon.

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EFFECTS OF ARTERIAL CARBON DIOXIDE TENSION AND OXYGEN SATURATION ON CEREBRAL BLOOD FLOW AUTOREGULATION IN DOGS

by

EGIL HÄGGENDAL and BÖRJE JOHANSSON

ABSTRACT

EGIL HÄGGENDAL and BÖRJE JOHANSSON *Effects of arterial carbon dioxide tension and oxygen saturation on cerebral blood flow autoregulation in dogs* Acta physiol scand 1965 66 Suppl 258 27—53 — Pressure flow relationships of the cerebral circulation under influence of variations in arterial carbon dioxide tension and oxygen saturation were studied in pentobarbital anaesthetized dogs Cerebral blood flow was measured by recording of the γ emission of radioactive krypton (Kr^{85}) which was injected into the vertebral artery The recorded desaturation curve was resolved into two exponential phases and the fast phase was considered as representative of blood flow in the grey matter of the brain It was demonstrated that autoregulation of flow occurred within wide pressure limits under normo and hypocapnia Autoregulation was less pronounced under hypercapnia and was abolished at low arterial oxygen saturation Possible mechanisms of autoregulation of cerebral blood flow are discussed Evidences are presented which speak against the theory that changes in the carbon dioxide tension are the main cause of this autoregulation The results from previous studies concerning the principal effects of arterial carbon dioxide tension and oxygen saturation on cerebral blood flow were confirmed

INTRODUCTION

Autoregulation of blood flow can be defined as an inherent tendency of an organ or a vascular circuit to maintain the blood flow unchanged in spite of variations in perfusion pressure.

In the earlier part of this century it was generally assumed that cerebral blood flow was almost exclusively dependent on the level of the arterial blood pressure which would imply absence of autoregulation in this tissue. The relative constancy of the blood flow through the brain was ascribed to the homeostatic regulation of blood pressure (for ref. see FOG 1934, WOLFF 1936 and LASSEN 1959). An active regulation of cerebrovascular tone was demonstrated, however, in 1928 by FORBES and WOLFF using the pial window technique of FORBES. FOG (1934 and 1939) found that the pial arterioles contracted when systemic blood pressure increased and dilated with pressure reductions regardless of the method used for varying the blood pressure. These reactions were not altered after sectioning the vagal, carotid sinus and cervical sympathetic nerves and FOG suggested that the pressure changes *per se* were responsible for the vascular reactions. Different techniques for measurement of cerebral blood flow have been used in more recent investigations and the results have shown that autoregulation does occur in the brain circulation (HAFKENSCHIEL *et al.* 1951, McCALL 1953, CARLYLE and GRAYSON 1955, HARPER 1963, PAPELA and GREEN 1964, see also LASSEN 1959 and 1964).

The mechanisms by which autoregulation is effected in different vascular regions are still a matter of dispute. The importance of "myogenic", "metabolic" and "mechanical" factors for producing autoregulation of blood flow in various tissues was discussed in a recent symposium (BERNE 1964, FOLKOW 1964, JOHNSON 1964). FOG's interpretation of his results, referred to above, would rather be in conformity with the myogenic theory of autoregulation. As to the importance of local chemical factors in the control of cerebral circulation particular attention may be paid to the oxygen and carbon dioxide tensions since blood flow through the brain is markedly influenced by variations of these parameters in the arterial blood. Hypercapnia and hypoxia produce cerebral vasodilatation while hypocapnia and hyperoxia are associated with vasoconstriction (SCHMIDT 1928, WOLFF and LENNOX 1930, KETY and SCHMIDT 1948 b, for further ref. see LASSEN 1959). Any decrease of blood pressure will

primarily be associated with a tendency to decreased flow which will raise the local carbon dioxide tension and lower the oxygen tension This would in turn cause cerebral vasodilatation and thereby a tendency to recovery of flow The reverse would happen with a primary increase in blood pressure In this way local chemical factors will tend to produce blood flow autoregulation It seems as if some information on the importance of this latter mechanism might be obtained by studying the ability of the cerebral circulation to maintain autoregulation under abnormal conditions of arterial O and CO_2 tensions The purpose of the present experiments was to study the effects of changes in perfusion pressure on the cerebral blood flow of dogs subjected to such changes in the arterial blood gas levels

METHODS

In the present study 28 mongrel dogs with body weights varying between 7 and 16 kg were used Of this total 4 experiments had to be excluded either because the animals died or deteriorated at an early stage or because technical difficulties occurred Thus the results are based upon 24 dogs in which 235 measurements of cerebral blood flow were performed

The material was subdivided into six groups based upon the blood gas situation when the measurements of cerebral blood flow (CBF) were done (Table I) The first three groups comprise variations in arterial carbon dioxide tension (P_{aco_2}) but the arterial oxygen saturation (S_{ao_2}) was here kept above 90 per cent The next three groups contain besides the variations in P_{aco} reductions in S_{o_2} The arterial blood pressure was varied in the same way and to the same degree in each of the six groups

Group A This group of 16 animals was ventilated with air at a normal rate and tidal volume and 56 measurements of CBF were performed at different arterial blood pressures P_{co_2} was between 30 and 50 mm Hg S_{o_2} was 90 per cent or more

TABLE I Subdivision of the material

Group	A	B	C	D	E	F
Number of animals	16	11	[illegible]	[illegible]	5	8
Number of CBF measurements	56	49	[illegible]	34	14	30
P_{co_2} mm Hg	30—50	<30	>50	30—50	<30	>50
S_{o_2} per cent	>90	>90	>90	<90	<90	<90
Results in table	II	III	IV	V	V	V

Group B This group consists of 49 measurements of CBF on 11 hyper ventilated dogs at different arterial blood pressures P_{aCO_2} was below 30 mm Hg S_{aO_2} was more than 90 per cent

Group C In this group 52 measurements of CBF were performed at different arterial pressures on 12 dogs which were ventilated with gas mixtures con taining 5—10 per cent of carbon dioxide P_{aCO_2} was more than 50 mm Hg S_{aO_2} as in the groups A and B was 90 per cent or more

Group D In 9 dogs 34 determinations of CBF were done at different blood pressures The animals in this group had about the same ventilation as the animals in the group A but were ventilated with mixtures of low oxygen content P_{CO_2} was as in group A between 30 and 50 mm Hg but S_{aO_2} was less than 90 per cent

Group E In 5 dogs which were hyperventilated with gas mixtures of low oxygen content 14 measurements of CBF were performed at different arterial blood pressures P_{aCO_2} was as in group B below 30 mm Hg S_{aO_2} was below 90 per cent

Group F This group consists of 30 measurements of CBF at different arterial blood pressures on 8 dogs which were hypoventilated usually with air some times with high CO_2 or low O_2 mixtures P_{aCO_2} was here as in group C more than 50 mm Hg S_{aO_2} was less than 90 per cent

The animals were anaesthetized with pentobarbital intravenously in a dose of 25—30 mg/kg body weight During the experiment small doses of pento barbital (2—3 mg/kg body weight) were given at intervals of about two hours in order to keep the anaesthesia at a relatively constant level As the successful experiments lasted about six to seven hours a total of 30—40 mg pentobarbital per kg body weight was used Immediately after induction of anaesthesia a tracheal tube was inserted to avoid respiratory complications and to permit mechanical ventilation The body temperature as measured in the rectum was maintained at 37—38° C with the aid of an electrical heating pad Clotting was prevented by intravenous administration of heparin

After completion of the operative preparation the animals were curarized with gallamine triethiodide (Flaxedil M & B) 1 5—2 5 mg/kg body weight as initial dose and during the experiment 0 5—1 0 mg/kg as supplementary doses After curarization ventilation was given via the tracheal tube by means of an adjustable pump Alterations in blood gas composition i e P_{CO_2} and S_{O_2} were produced by increasing or decreasing the ventilation or by ventilating the animals with different gas mixtures After every change in ventilation 30 minutes were allowed to elapse before samples for blood gas analysis were taken or measurements of CBF were performed Under the prevailing conditions i e after 30 minutes of unchanged mechanical ventila

tion repeated measurements of P_{aCO_2} showed this value to be very constant for several hours (HAGGENDAL, NILSSON and NORBÄCK 1965).

Arterial blood was sampled from the abdominal aorta through a plastic tube inserted through a femoral artery. When cerebral venous blood was to be taken a needle was bored through the skull about 3 cm rostrally to the occipital protuberance into the superior sagittal sinus. The samples were drawn by very gentle sucking. Oxygen saturation was determined according to the method of S. STENHAGEN (HOLMGREN and PERNOW 1959, BJURE and NILSSON 1965).

The hemoglobin concentration was determined spectrophotometrically according to DRABKIN and AUSTIN (1935). The oxygen capacity was calculated on the basis of the hemoglobin determination on the arterial samples. The same samples were also used for determination of the arterial pH and carbon dioxide tension according to the micromethod of SIGGAARD ANDERSEN *et al.* (1960). Corrections of the measured pH with regard to oxygen unsaturation were considered unnecessary.

The arterial blood pressure was recorded from the catheter in the femoral artery with a transducer of the inductance type (Elema) feeding into an amplifier unit which in turn was coupled to a direct writing Mingograf (Elema). Mean pressure was obtained by electric integration. The horizontal plane through the entrance of the catheterized vertebral artery into the skull was taken as the reference level. Variations in arterial blood pressure, which was considered as representative of cerebral perfusion pressure, were produced by varying degrees of controlled bleeding through the femoral artery into a reservoir maintained at 37° C, from which the blood could be reinfused.

Determinations of CBF were made according to the principles of a method developed by LASSEN *et al.* (1963). This method is based upon the intra-arterial administration of a radioactive indicator (Kr^{85}) to the cerebral tissue and external measurement of the disappearance of the indicator, the γ emission of which is registrated. A thin polyethylene catheter (PE 110 or 160) was inserted from a small side branch of the subclavian artery and advanced 5—8 cm up into the left vertebral artery, the origin of which had been exposed. The catheter was filled with a heparin saline solution and sluiced at intervals. The isotope Kr^{85} (Radiochemical Centre, Amersham, England) was dissolved in saline (0.9 per cent NaCl). The volume of Kr^{85} solution injected for each determination of CBF was 1—3 ml, and the injections lasted about 3 sec. The γ activity was measured by means of a scintillation detector coupled to a ratemeter (Nukleoninstrument AB, Göteborg), the time constant of which was set at 1 sec. The detector was placed over the skull so that most of the left hemisphere was seen by the crystal. The influence of extracerebral

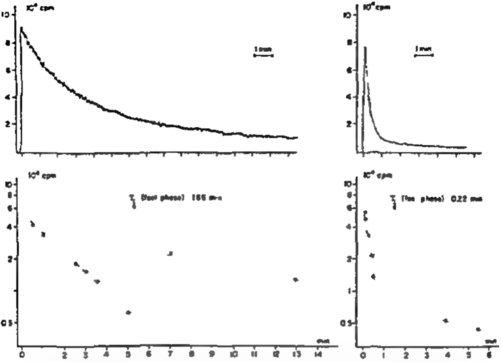

Fig 1 Above Original recording of Kr^{85} clearance in hypocapnia (left) and hypercapnia (right) at a mean arterial blood pressure of 80 mm Hg Below Corresponding semilogarithmic plottings with the two monoexponential phases CBF is calculated from the $T\frac{1}{2}$ values according to CBF ml/(100 g min)$=100\ \dfrac{0.693 \cdot S}{T\frac{1}{2}}$ 0.95 has been used as tissue blood partition coefficient (S) for the fast component

activity was reduced as much as possible by lead collimation and the expired air was carried off from the dog through a tube into the outer air The γ activity was recorded on a potentiometer writer (Varian recorder with the paper speed of 1 inch/1.5 min)

After semilogarithmic plotting the clearance curves recorded for 10—15 minutes were resolved into two exponential phases (Fig 1) The resolution proved easy to carry out since the fast phase generally had a flow 5—10 times larger than the slow one Despite careful collimation some activity was recorded from extracerebral tissue and at the end of the experiment this activity was rather high In the semilogarithmic curves this extracerebral flow could be resolved out as a third very slow phase and so corrected for (HAGGENDAL *et al* 1965) The fast phase of the clearance curve has been taken as an expression of the blood flow in the most rapidly perfused parts

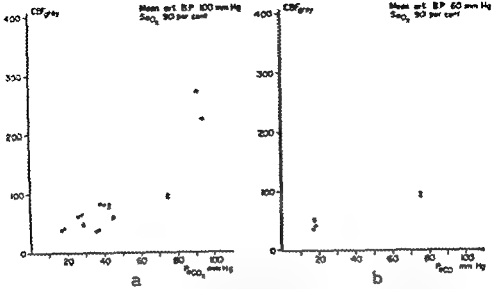

Fig 2 a Influence of arterial carbon dioxide tension on CBF during arterial normotension (100 mm Hg) and arterial oxygen saturation above 90 per cent (The values are from Group A, B and C) The correlation curve is rather steep when P_{CO_2} is raised over about 50 mm Hg indicating the marked vasodilating effect of hypercapnia

Fig 2 b Influence of arterial carbon dioxide tension on CBF during hypotension (60 mm Hg) and arterial oxygen saturation above 90 per cent (The values are from Group A, B and C) In the higher range of P_{CO_2} the effect is somewhat less pronounced than in Fig 2 a due to the dilatation produced by the hypotension

of the brain i e most likely in the grey matter The slower phase would correspond to the blood flow in other parts of the brain i e mainly in the white matter (cf LASSEN *et al* 1963) Experimental evidences supporting this interpretation of the composite clearance curve are obtained by HAGGENDAL *et al* (1965)

In the present study the influences of blood pressure and blood gas content only on the fast phase were examined The half time of decay (T½) for this component of the clearance curve is given with an accuracy of approximately 1 sec

RESULTS

The results are presented according to the subdivision of the material into the six groups which were based upon the induced changes in blood gas composition (Table I)

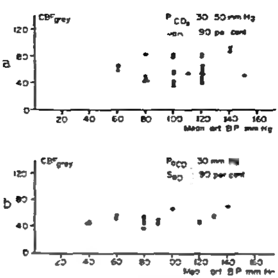

Fig 2a CBF in relation to mean arterial blood pressure in animals with arterial carbon dioxide tension of 30–50 mm Hg and arterial oxygen saturation above 90 per cent (Group A) The correlation indicates autoregulation of flow within the whole pressure range

Fig 2b CBF in relation to mean arterial blood pressure in hypocapnic animals with arterial oxygen saturation above 90 per cent (Group B) The correlation indicates autoregulation of flow within the whole pressure range

Effect of P_{aCO_2} on CBF during normo and hypotension

The results presented in Fig 2 a are based upon flow values from dogs in Group A, B and C during normotension i e a mean arterial blood pressure of 100 mm Hg Fig 2 a shows that when P_{aCO_2} was raised over 50 mm Hg there was a very strong dilating effect on the cerebral vessels so that at tensions about 80 mm Hg CBF was increased by about 100 per cent whereas tensions below 30 mm Hg had only slight effect on CBF From Table III it can be seen however that when the same dog was hyperventilated to different degrees it showed rather marked variations of CBF for instance dog B13 which had a reduction of CBF of about 25 per cent when P_{CO_2} was reduced from 26 to 17 per cent In hypotensive dogs there was also such an influence of P_{aCO_2} on CBF (Fig 2 b) but the effects were somewhat less pronounced at high carbon dioxide tensions indicating some degree of dilatation of the vessels due to the blood pressure reduction.

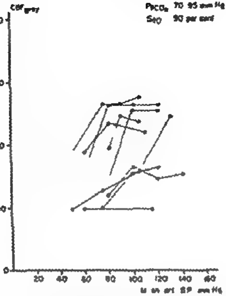

Fig 4 Pressure flow relationship in hypercapnic animals with arterial oxygen saturation above 90 per cent (The values are from Group C) Autoregulation is present in the higher pressure range in all animals except one (which is represented with open circles)

Pressure flow relationships at different levels of P_{aCO_2}

The pressure flow relationships were examined during normo hypo and hypercapnia Dogs with P_{aCO_2} in and rather close to the normal range — Group A Table II — had CBF at an almost constant level in spite of great variations in blood pressure (Fig 3 a) From Table II it is evident that most animals showed this flow autoregulation within a pressure range from 50 or 60 to over 150 mm Hg while some animals showed a reduction in CBF when blood pressure was lowered below 80 mm Hg Dogs A9 and A11 had this marked constancy in CBF even with blood pressure reductions to very low levels

CBF autoregulation was also observed in the hyperventilated dogs of Group B — Table III (Fig 3 b) Some animals in this group maintained the flow unchanged when the mean arterial pressure was reduced even to 50 or 40 mm Hg This fact is well illustrated by dogs B12 and B15

When hypercapnia had been induced — Group C Table IV — the tendency to autoregulation was less pronounced Due to variations in P_{aCO_2} the flow values in this group are distributed over a wide range The pressure flow relationships are therefore represented by lines through the points for each individual experimental dog Of the 9 dogs in Fig 4 there was only one which showed a merely passive pressure flow relationship the others maintained the flow fairly unchanged until blood pressure was reduced below 100 to 80 mm Hg In this group there are 3 dogs which are not presented in Fig 4

TABLE II Cerebral hemodynamics and blood parameters in normocapnic animals (Group A)

Exp dog	CBF ml/(100 g min)	Mean art B P mm Hg	P_{aCO_2} mm Hg	pH_a	S_{aO_2} per cent	S_{vO_2} per cent	O_2 cap ml/100 ml	$(a-v)_{O_2}$ ml/l	CMR_{O_2} ml/(100 g min)	CVR mm Hg / ml/(100 g min)
A1a	53	1[illegible]0	36	7 35	94		17 0			2 8
b	47	80								1 7
c	[illegible]	[illegible]0								0 9
A4a	66	120	36	7 41	90		21 3			1 8
A[illegible]a	[illegible]	130	37	7 41	93		18 0			2 1
A6a	65	120	45	7 35	92		17 6			1 8
b	63	70								1 1
c	8	100								1 7
d	[illegible]	120								1 0
A8d	93	90	38	7 40	[illegible]		16 6			1 1
e	66	60								0 0
f	83	120								1 4
A9a	94	140								1 5
b	89	10[illegible]	43		93	27	13 6	91	7 9	1 [illegible]
c	[illegible]3	75								1 4
d	8[illegible]	160								2 0
A10a	78	1[illegible]0	43	7 40	94	[illegible]1	19 3	79	6 1	3 5
b	73	80								1 1
c	79	100								1 3
d	70	190								2
A11a	98	140	40	7 41	92	[illegible]2	16 4	6[illegible]	7	1 0
b	92	100								1 2
c	[illegible]7	70								1 [illegible]
d	78	150								1 9
A14a	74	12[illegible]	31	7 51	92	[illegible]	1[illegible] 9	97	7 2	1
b	93	80								1 0
c	78	[illegible]								0 7
d	74	13[illegible]								1 8
A17a	40	130	37	7 40	93		23 8			3 3
b	[illegible]	100								2 6
c	44	[illegible]								1 8
d	41	120								2 9
l	[illegible]	140	36	7 [illegible]6	93					3 0
m	37	100								2 [illegible]
n	30	80								2 [illegible]
o	43	120								[illegible]

119a	4	1 0	41	7 39	11	14	-- 0	13	3 1	4 0
b	40	1?,								3 1
c	43	100								2 3
120b	5,	1_0	33	34	),	40	23 0	111	0 1	2 2
121a	6,	12,	49	7 39	[illegible]	6?	13 7	62	3 4	1 9
b	65	60								0 9
c	5	100								1 9
d	51	80								1 6
e	0	1?0								2 4
k	35	110	4,	7 31	94	11	1, ,	66	3 0	-0
l	57	80								1 4
m	40	60								1 0
n	63	11,								1 0
o	13	40								0 9
12 a	44	1 0	31	7 39	91	30	17 8	107	4	2
b	43	90								1 9
c	43	60								1 4
d	40	110								2 8
1 3a	35	170	30	7 37	94	2				2
124a	60	190	39	7 38	91	?	17 5	67	4 0	2 0

Exp dog = Experimental dog. The capital letters A–F indicate to which group the actual measurements belonged. The figure after the capital letter indicates the number of the experimental dog. It is evident that the same animal appears in different groups. The small letters after the figure indicate the chronological order of the measurements.
CBF = The calculated blood flow of grey matter in ml/(100 g min)
Mean art BP = Mean arterial blood pressure in mm Hg
P_{CO_2} = Arterial carbon dioxide tension in mm Hg
pH_a = Arterial pH
S_{O_2} = Arterial oxygen saturation in per cent
S_{O_2} = Oxygen saturation in per cent in venous blood from superior sagittal sinus
O_2 cap = Oxygen capacity of the hemoglobin in blood in vol per cent
$(a-v)_{O_2}$ = Arterio venous oxygen difference. Venous oxygen content here refers to blood from superior sagittal sinus
CMR_{O_2} = Cerebral metabolic rate of oxygen calculated from CBF and $(a-v)_{O_2}$ and expressed in ml/(100 g min)
CVR = Cerebrovascular resistance calculated from mean art BP and CBF and expressed in mm Hg/(ml/100 g min)

TABLE III Cerebral hemodynamic and blood parameters in hypocapnic animals (Group B)
Abbreviations are explained in the legend of Table II

Exp dog	CBF ml/(100 g min)	Mean art BP mm Hg	$P_{a_{CO_2}}$ mm Hg	pH_a	$S_{a_{O_2}}$ per cent	$S_{v_{O_2}}$ per cent	O_2 cap ml/100 ml	$(a-v)_{O_2}$ ml/l	CMR_{O_2} ml/(100 g min)	CVR mm Hg ml/(100 g min)
B3a	[illegible]	150	30	7.41	91		14.4			1.8
b	1	1 0								1
c	60	140	[illegible]	[illegible]	93		14			[illegible]
B6e	0	1 0	[illegible]	[illegible]	90		13.6			[illegible]
f	0	100								1.8
g	33	00								1.1
h	43	40								0.0
B8a	61	83	[illegible]	[illegible]	[illegible]		16.3			1.3
b	60	[illegible]								1.6
c	57	60								1.1
B9e	50	140	[illegible]	7.33	[illegible]	13	13.8	1.0	6.6	[illegible]
f	50	110								[illegible]
g	0	60								1
h	00	33								[illegible]
i	60	100	28	7.4	98	[illegible]	13.0	123	7.3	1.3
j	60	[illegible]								1.2
k	71	140								1.9
B10h	47	103	1[illegible]	7.63	98	[illegible]	17.4	1[illegible]8	0.1	2.8
i	33	80								1.3
j	31	60								1
l	41	1 0								3

B11e	37	1..	1	7 0	1.	19	17 4	137	1	2 8
f	40	110								1 6
g	37	[illegible]								0
h	30	180								2 0
B1_	4.	30	4	7 8	98	33	17 0	101	4 0	1 7
f	41	70								0 0
g	44	40								[illegible]
h	37	80								1
B13	74	110	.0	58	100	.4	1 5	0	5	1 6
b	.8	1.								1 1
c	74	80								0 8
d	74	60	17	7 65	98	49	14 8	73	4 2	2 2
i	.8	130								1 5
j	5.	80								1 1
k	1	[illegible]								0 0
l	44	40								4
m	.	170								0
B1k	11	115	27	7 5.	0	29	14 0	93	1 7	2 2
l	37	.0								1 3
m	40	0								3 3
n	30	130								1
B16d	6.	1.0	.9	7 44	93	1	19 5	8_	4 7	1 6
e	40	80								2 0
f	44	90								0
g	47	120								4 0
B18j	40	180	18	7 43	97		22 8			1 9
k	45	80								1 4
l	44	60								

TABLE IX Cerebral hemodynamics and blood parameters in hypercapnic animals (Group C)
All results are explained in the legend of Table II

Exp. dog	CBF, ml/(100 g min)	Mean art. BP mm Hg	P_{aCO_2}, mm Hg	pH_a	S_{aO_2}, percent	S_{vO_2}, percent	O_2 cap ml/100 ml	(a-v) O_2, ml/l	CMR_{O_2}, ml/(100 g min)	CVR mm Hg/ml/(100 g min)
C4h	127	75	0	15	91					0.6
i	217	130								0.6
j	157	105								0.7
k	84	50								0.5
l	111	100								0.8
C10e	95	105	95	7.05	97	50	11	18	1.0	0.4
f	103	80								0.4
g	44	90								0.4
C11i	75	130	51	7.24	98	10	7.0	41	3.0	1.8
j	00	70								1.2
k	88	130								1.5
l	44	40								0.9
C12a	98	115	5	4	91	8	1.0	10	1.8	1
b	98	80								0.6
c	91	110								1.2
d	94	90								0.6
C13e	0	110	94	7.05	99	5	10.2	17	1.4	0.5
f	33	80								0.9
g	188	90								0.9
h	90	115								0.4
C14e	213	105	91	7.09	97		1.1			0.4
f	91	75								0.9
g	188	50								0.3
h	81	90								0.9

C1 a	366	130	100	7 1	19	9_	14 9	10	3	0 4
b	-45	10								0 4
c	199	55								0 3
d	146	40								0 3
e	300	9								0 3
f	389	130								0 3
C17e	154	140	74	7 13	93		-1 9			0 9
f	1 0	80								0 7
g	165	100								0 6
h	148	120								0 8
C18 a	88	165	6	7 1	100		23 9			1 9
b	83	60								1 0
c	100	100								1 0
d	9_	170								1 3
e	13	100								1 6
C10f	-54	1 0	94	7 07	96	84	-7 4	0	7 4	0 5
g	-54	100								0 4
h	147	80								0 5
i	? 0	1 0								0 6
C20e	03	1 0	8?	7 00	97	80	21 6	-4	8	0 ?
d	263	100								0 4
	263	80								0 3
f	157	60								0 4
g	263	100								0 4
C??a	165	170	77	7 07	97	9	17 9	33	5	0 7
f	154	100								0 6
g	100	75								0 8
h	165	1 3								0 8

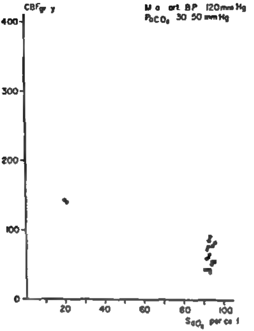

Fig 5 Influence of arterial oxygen saturation on CBF during normotension (120 mm Hg) and arterial carbon dioxide tension between 30 and 50 mm Hg (The values are from Group A and D) With pronounced hypoxia the vasodilating effect is evident and it can be seen that CBF is increased by about 100 per cent when S_{aCO_2} is reduced from 90 to 20 or 30 per cent

because their hypercapnia was either too little or too much pronounced to be considered as representative Dogs C11 and C18 had P_{aCO_2} of 51 and 56 mm Hg respectively and a rather high vascular tone with relatively low CBF and a marked autoregulating capacity Dog C15 showed no signs of flow autoregulation but as it had the highest P_{aCO_2} of the group (100 mm Hg) it was also excluded from Fig 4

Effect of S_{aO_2} on CBF during normotension

Fig 5 shows how CBF was influenced by various degrees of arterial oxygen saturation *per se* *i e* when arterial blood pressure and P_{aCO_2} were kept at a stable level In Fig 5 the flow values are presented from the dogs with P_{aCO_2} of 30—50 mm Hg (Groups A and B) at blood pressure of 120 mm Hg It can be seen that CBF increased about 100 per cent when S_{aO_2} was lowered from about 90 to 20 or 30 per cent Within the higher range of oxygen saturation Fig 5 shows a great spread of the flow values Single experiments where reductions of S_{aO_2} in this range were induced and P_{aCO_2} unchanged showed

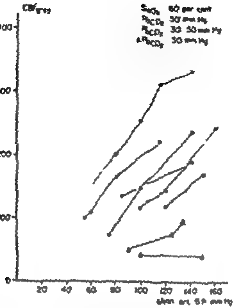

Fig 6 Pressure flow relationship in animals with arterial oxygen saturation below 60 per cent at different levels of arterial carbon dioxide tension (The values are from Group D E and F) Most animals show a passive pressure flow relationship

however obvious increases of CBF An example of this is dog 10 (Table I and V) which increased the flow about 30 per cent when S_{ao_2} was reduced from 91 to 82 per cent

Pressure flow relationships during arterial hypoxia

The results are given in Table V which contains Groups D E and F When S_{ao_2} was reduced to less than 60 per cent most animals showed a merely passive pressure flow relationship (Fig 6) Each individual experimental animal is presented in this figure for the same reason as in Fig 4 Only during simultaneously provoked hypocapnia (Group E) was it possible to demonstrate a constant CBF during variations in blood pressure Also when the hypoxia was less pronounced i e when S_{ao_2} was between 70 and 90 per cent the tendency to autoregulation seemed to be impaired as is indicated by dog 18 in Groups C and D This animal showed a more constant CBF at lower blood pressure during hypercapnia and while the arterial blood was fully oxygenated than it did during hypocapnia with S_{ao_2} of 71 per cent and thus in spite of lower cerebrovascular resistance under the former conditions Fig 6 illustrates further that the influence of P_{aco_2} on CBF is evident also under these conditions of reduced oxygen saturation

TABLE V Cerebral hemodynamics and blood parameters in animals with low arterial oxygen saturation (Group D, L and T). Abbreviation are explained in the legend of Table II

Exp dog	$CBF_{g\,v}$ ml/(100 g min)	Mean art B P mm Hg	P_{CO_2} mm Hg	pH	S_{aO_2} per cent	S_{vO_2} per cent	O_2 cap ml/100 ml	$(a-v)_{O_2}$ ml/l	CMR_{O_2} ml/(100 g min)	CVR mm Hg / ml/(100 g min)
D?a	54	130	32	7 4	97		12 0			1 4
b	71	75								1 1
e	83	130	31							1 6
f	75	65		7 30						0 9
g	57	35								0 6
D7a	94	100	42	7 38	83		1? 9			1 1
b	98	100								1 1
c	68	75								0 9
d	94	100								1 1
D14i	188	140	33	7 39	53		1?			0 ?
j	134	85								0 6
D15g	235	140	40		21	10	14 6	16	3 8	0 6
h	147	100								0
i	73	75								1 0
j	178	140								0 8
D18m	244	160	45	7 1	90		28 0			0
n	118	100								0 8
o	366	200								0 5
p	143	190								0 9
D10d	54	75	42	7 29	8	47	22 ?	78	4 2	1 4
e	[illegible]	135								2
D20a	72	120	43	7 37	80	69	17 9	37	3 3	1 7
b	73	95								1 3
D23b	105	150								0 9
c	120	120	37	7 30	33	12				1 0
d	80	120	35	7 5	4	34				1 4
e	82	120	36	7 ?6	69	45				1
f	[illegible]	120	38	7 ?7	87	?8				1 9
g	160	120	37	7 ?	36	?2				0 9
D24b	140	120	44	7 18	21	12	19 0	19	2 5	0 9
m	92	190	41	7 20	17	20	19 3	?3	4 9	1 3
n	8?	120	4?	7 ?1	74	3	14 4	30	–	1 4
o	106	120	40	7 18	?	23	1? 1	8	0 9	1 1
f	101	190	41	7 14	34	17		?2	1 3	1
E3e	[illegible]	9?	?	7 30	87		13 3			1 0
i	?3	65								1 0

F18a	97	135	_>	7 41	49	0	_1 0	00	~4	1 4
h	0	90								1 8
c	5	1_5								1 7
I1i	3	140		7 47	61		_11			_6
j	_5	100								1 8
k	_0	80								1 6
I18f	0	18	6	7 41	71		22 7			3 1
g	0	1 0								_1
h	47	100								_1
i	80	180								_
I19j	41	150	29	7 38	73	26	_2 9	62	2 5	3 -
k	41	100								2 4
F1d	140	140	62	7 09	19		17 8			1 0
e	68	0								0 7
F_c	263	130	71	7 10	30		13 8			0 _
d	132	45								0 3
I4b	188	110	80	7 15	79		20 6			0 6
c	76	80								1 1
d	218	11								0 _
e	265	130								0
f	73	60								0 8
F b	220	11>	90	7 05	58		19 3			0 5
c	165	80								0 5
d	110	60								0 5
e	100	75								0 6
f	188	1_5								0 7
g	77	110	77	7 17	70		18 6			1 0
h	43	0								1 2
I7e	88	0	84	7 10	77		15 1			0 8
f	1_7	105								0 8
g	146	110								0 9
I10h	147	1 0	78	6 91	12	10	23 -	6	0 8	1 0
i	110	100								0 9
I21f	330	140			10	34	10 6	5	1 7	0 4
g	310	115								0 4
h	_0	100								0 4
i	200	80								0 4
j	148	60								0 4
I2?i	2_0	180	1 3	6 83	80	65	17 5	27	6 2	0 8
j	10,	120								0 -
k	177	1 0								0 8
l	115	100								0 9

DISCUSSION

The main purpose of the present investigation was to study the pressure flow relationships of the cerebral vascular bed under conditions of induced changes in the arterial carbon dioxide tension and oxygen saturation. The choice of method for determination of CBF seemed therefore to be of great importance since traumatizing procedures might interfere with the normal vascular reactions in the brain. The inert gas technique of KETY and SCHMIDT (1945, 1948 a) is a non traumatic method which permits measurement of blood flow in the intact brain but it is relatively time consuming and requires much blood sampling, about 30 ml for one determination of CBF. This seemed to be less suitable for the present investigation where 10—15 blood flow determinations were to be done in each experiment. Regional cortical blood flow was studied by LASSEN and INGVAR (1961) by external recording of β activity over the exposed cerebral cortex during wash out of intra arterially administered Kr^{85} (See also INGVAR and LASSEN 1962.) For the present study the corresponding method with γ recording (LASSEN *et al* 1963) was preferred in order to leave the brain as intact as possible. The surgical trauma associated with the technique is minimal and no blood samples need to be taken for the flow measurements.

Analyzed in the way here described the technique yields separate values for blood flow in two different compartments of the brain, one with high and one with low perfusion rate. The present study is concerned with the reactions of the fast component only. This choice is connected with the fact that this component has been assumed to represent blood flow in the grey matter (cf LASSEN and INGVAR 1961, INGVAR and LASSEN 1962, LASSEN *et al* 1963), an assumption for which experimental evidence has been obtained by HAGGENDAL *et al* (1965) by recording clearance curves from local micro injections of Kr^{85} and by comparing simultaneous recordings of γ and β radiation after intra arterial administration of the isotope. The blood flow of the grey matter was considered to be of the main interest for the problems which are discussed in this study. Further, it was desirable to obtain as many recordings of CBF as possible from the same animal in different states. This necessitated the use of short observation times (10—15 minutes). Under these circumstances only the fast component can be determined accurately, whereas the value for the second component is determined with a considerable inaccuracy, especially when extracerebral activity is high (HAGGENDAL *et al* 1965).

The vertebral artery was chosen for the isotope injections because this artery supplies less extracerebral tissue than the internal carotid in the dog (SCHMIDT 1950, p 11).

For the calculation of tissue blood flow from the Kr^{85} wash out curves a partition coefficient between cerebral cortex and blood of 0 95 (LASSEN and MUNCK 1955 INGVAR and LASSEN 1962 GLASS and HARPER 1962) has been used throughout the present study This value is to some extent dependent upon the hematocrit level but this has not been systematically determined in these experiments However since hemoglobin concentration which was repeatedly measured showed only moderate variations during the course of each experiment values of CBF obtained in one and the same animal should be affected to about the same extent

As demonstrated in several investigations the anaesthesia employed influences the cerebral blood flow and reduces the oxygen uptake (WECHSLER DRIPPS and KETY 1951 HOMBURGER *et al* 1946) In some of these studies there has been no corresponding decrease in CBF but this might be due to concomitant hypercapnia as pointed out by LASSEN (1959) Differences in the depth of anaesthesia probably contribute to the disparate flow values in different animals On the other hand the level of anaesthesia probably remained relatively constant throughout each experiment as indicated by the constancy of the oxygen consumptions (Tables II—IV)

The calculations of oxygen uptake in the cerebral cortex are based upon two major assumptions Firstly it is assumed that blood flow estimated from the fast phase of the two compartment system belongs to a homogeneously perfused cerebral cortex and secondly cortex is assumed to be the predominant source of venous blood in the sagittal sinus The latter assumption has been used in previous investigations on cortical metabolism but diploic anastomoses were eliminated in some of these studies The measurements of oxygen uptake made in the present experiments were mainly intended as an indicator of possible changes in the condition of the animals The mean value of 5 3 ml/100 g min resulting from 27 determinations is in reasonable agreement with the values of 7 0 and 5 9 obtained by GLEICHMANN *et al* (1962) and by HOMBURGER *et al* (1946) with and without elimination of diploic veins respectively In accordance with previous studies (GIBBS MAXWELL and GIBBS 1947 KETY and SCHMIDT 1948 b) oxygen uptake was found to be almost independent of variations in CBF induced by alterations in P_{CO_2} (Tables II—IV)

Carbon dioxide is known to be a strong vasodilator in the brain and according to KETY and SCHMIDT (1948 b) hypercapnia induced by inhalation of 5 to 7 per cent CO_2 increases CBF by about 75 per cent while hyperventilation decreases CBF by approximately 60 per cent when P_{aCO_2} is reduced to 25 mm Hg A recent study by REIVICH (1964) gives a detailed report on arterial CO_2 tension and cerebral hemodynamics In his experiments P_{CO_2} was varied

from [illegible] to over 400 mm Hg and maximal increase in blood flow was reached at about 150 mm Hg. The present results concerning the influence of CO_2 during normotension as illustrated in Fig. 2 are in good agreement with the earlier investigations. A similar but less pronounced effect of CO_2 was found in hypotensive animals (Fig. 3). According to HARPER (1963) variations in P_{aCO_2} do not affect CBF when perfusion pressure is reduced to about 50 mm Hg, since the cerebral vessels are then already markedly dilated due to hypotension.

The induced acute changes of P_{aCO_2} were of course associated with changes in blood pH (see Tables II—V). No attempts have been made to investigate the influence on CBF of the pH variations *per se*. According to HARPER and BELL (1963) there is however no significant influence of metabolic acidosis or alkalosis on cortical blood flow in dogs (see also LASSEN 1959).

Low oxygen content of the arterial blood is known to cause an increased CBF (KETY and SCHMIDT 1948 b). Supranormal oxygen tension has been reported to have the converse effect (KETY and SCHMIDT 1948 b) but some recent investigations (LAMBERTSEN *et al.* 1959, LAMBERTSEN 1961 and REIVICH 1964) have indicated that CBF is not influenced by variations in P_{aO_2} above the range of 50 to 60 mm Hg. The present results (Fig. 5) are in agreement with the previous studies concerning the effect of reduced oxygen saturation. No attempts have been made to evaluate the results in terms of changes in oxygen tension. Reduction in oxygen saturation has apparently a weaker dilator action on cerebral vessels than hypercapnia, at least within the limits used in this study.

In accordance with earlier studies (HARPER 1963, RAPELA and GREEN 1964, see also LASSEN 1959 and 1964) the present findings indicate that CBF remains almost constant in spite of even marked variations in blood pressure. This autoregulation of flow was most pronounced in animals with normal oxygen saturation during hypo- or normocapnia, but when P_{aCO_2} was raised to more than 90 mm Hg it was also possible to demonstrate a relative constancy of flow in the higher blood pressure range (above 80 to 90 mm Hg). The investigation by SAGAWA and GUYTON (1961) has indicated that autoregulation does not occur in the cerebral circulation. The reason for this discrepancy of results is not clear but it is possible that traumatic experimental procedures may interfere with the autoregulatory vascular mechanisms. In those of the present experiments where arterial oxygen saturation was markedly reduced there was mostly a merely passive relationship between perfusion pressure and CBF.

Blood flow autoregulation has also been demonstrated in other vascular circuits besides that of the brain, for instance kidney, skeletal muscle and

intestine (SELKURT 1946, FOLKOW 1949, JOHNSON 1960). The autoregulatory mechanism has been discussed for several years and, conceivably, various factors may be involved to a different extent in different organs. There are apparently three main theories which can be briefly summarized as follows (see also FOLKOW 1964, JOHNSON 1964). According to the *passive mechanical theory* a rise in arterial blood pressure causes an increased tissue pressure in the encapsulated organ so that flow resistance increases in the thin walled vessels due to compression from without (HINSHAW *et al.* 1959). The occurrence of the so called waterfall phenomenon in some vascular beds (PERMUTT and RILEY 1963) is another example of passive mechanical factors which could contribute to autoregulation, especially if perfusion pressure is altered by primary changes in venous pressure. *The metabolic theory* implies that the change in blood flow, which is initiated by the change in perfusion pressure, causes a change in the local concentration of some vasoactive agents, normally brought to or eliminated from the tissue by the blood stream. The shift in the concentration of these agents, nutrient or metabolic, would affect the vascular smooth muscle in an autoregulatory direction (for ref. see BERNE 1964). According to *the myogenic theory* the altered tension of the vascular walls, produced by changes in blood pressure, influences the inherent automaticity of the smooth muscle cells (BAYLISS 1902, FOLKOW 1962). The vasoconstriction resulting from increased intravascular pressure is supposed to be independent of nervous mechanisms since isolated smooth muscle preparations have been shown to increase their "spontaneous" activity when stretched passively (BULBRING 1955).

All these mechanisms may contribute to the autoregulatory ability of the cerebral vessels. It is possible, for instance, that the *passive mechanical factors* may become of some importance during the influence of gravitational forces or when venous pressure is raised, for instance, at straining. However, with primary arterial pressure changes such a type of mechanism seems to be of negligible importance. This is indicated by the present observations of only minor changes (5 mm Hg) in cerebral venous pressure and in cerebrospinal fluid pressure when the arterial blood pressure was varied over a wide range (100 mm Hg) (see also FORBES and WOLFF 1928).

In the light of the present study the possible role of the *metabolic theory* in cerebral blood flow autoregulation may be discussed with respect to carbon dioxide and oxygen. It is evident that changes in the tension of carbon dioxide markedly influence cerebral vascular tone, but there are some facts which make it difficult to accept this as the main autoregulatory mechanism. First of all, as indicated by previous studies (REIVICH 1964) and by the results presented in Fig. 2 and 3 above, the cerebral vessels are relatively insensitive

to changes in P_{aCO_2} at or especially below the normal level i e under the conditions where autoregulation is most pronounced Similarly, changes in S_{aO_2} around the normal range seem to have little influence on CBF (Fig 5) The arterial oxygen tension under the conditions of the present study was probably not altered to much higher a degree than was S_{aO_2} It is difficult however to judge the importance of changes in tissue O_2 tension as a mechanism contributing to autoregulation The fact that autoregulation is abolished under conditions of severe anoxia might mean that tissue oxygen is an essential factor in the control of cerebrovascular tone (*cf* below) A purely metabolic mechanism would however hardly be able to effect an absolute regulation but would only cause a deviation of the pressure flow curve towards the pressure axis The *myogenic mechanism* on the other hand would not be subjected to this limitation at least not if it operates by changes in the rate of myogenic activity induced by distension (*cf* FOLKOW 1962) Autoregulation due to myogenic responses would obviously be abolished if the vessels were maximally dilated The fact that some constancy of flow during limited pressure changes remained also at the highest level of P_{aCO_2} obtained in the present study (90—100 mm Hg) indicates that the active vascular tone was not totally abolished This seems to be in agreement with the findings of REIVICH (1964) according to which maximum dilatation is not reached until the P_{aCO_2} is raised to 150 mm Hg It is interesting to note that under the influence of severe hypoxia autoregulation is lost at blood flow levels which do not indicate maximum dilatation (Fig 6) This might suggest that the ability of the smooth muscle cells to respond to pressure changes is dependent on an adequate oxygen supply

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FROM THE DEPARTMENT OF CLINICAL PHYSIOLOGY UNIVERSITY OF GOTEBORG SWEDEN

EFFECTS OF SOME VASOACTIVE DRUGS ON THE VESSELS OF CEREBRAL GREY MATTER IN THE DOG

by

EGIL HÄGGENDAL

ABSTRACT

EGIL HÄGGENDAL *Effects of some vasoactive drugs on the vessels of cerebral grey matter in the dog* Acta physiol scand 1965 *66* Suppl 258 55—79 — The effects on cerebral circulation of intravenously infused pressor substances noradrenaline and metaraminol (Aramine MSD) and injected papaverine were studied in 11 mongrel dogs anaesthetized with pentobarbital Cerebral blood flow (CBF) was measured by external recording of the clearance of radioactive krypton (Kr^{85}) from the brain after short intracarotid injections Resolutions of the clearance curves into the main components allowed evaluation of blood flow of the cerebral grey matter as distinguished from flow of other cerebral or extracerebral tissues Previous studies concerning the major effects of the used pressor drugs and papaverine were confirmed but the effects were found to be much more pronounced for the grey matter than for the brain as a whole The vasoconstricting effect of the pressor drugs was observed also in hypotensive states It was demonstrated that cerebral autoregulation of flow still functioned during the increased vascular resistance produced by the pressor drugs Papaverine was also found to dilate the cerebral vessels while the latter were under the influence of the pressor drugs The effects of these substances were also obvious when slight hypoxia and/or hypercapnia were induced The mechanism of the earlier described differentiated effect of pressor drugs in normal and hypotensive state is discussed

INTRODUCTION

The effects of many drugs on the cerebral circulation are often different from their effects on other vascular circuits In his review of the action of drugs on cerebral circulation SOKOLOFF (1959) points out the physiological and pharmacological behaviour of the cerebral circulation is sufficiently unique that drug actions exerted on most other vascular beds can only rarely be assumed to be operating similarly in the brain

It is natural that special interest is directed to the effects on the cerebral vessels of those of the sympathomimetic drugs which are known to have the greatest effect on other vascular beds and which are commonly used in therapeutics e g in the treatment of hypotensive conditions This type of sympathomimetic drugs is most often used therapeutically to improve the systemic circulation in hypotensive states by virtue of its pressor effects on the assumption that these drugs change the distribution of cardiac output in favour of the brain and the heart The action on cerebral blood flow and vascular resistance of such vasoactive substances noradrenaline and a synthetic vasopressor amine metaraminol (Aramine MSD) has been studied in a few earlier papers (KING SOKOLOFF and WECHSLER 1951 SENSENBACH MADISON and OCHS 1953 MOYER MORRIS and SNYDER 1954) Briefly summarized the observed effects after intravenously or intramuscularly administered pressor substances were an increase of cerebrovascular resistance (CVR) and a decrease of cerebral blood flow (CBF) In these studies the flow values were calculated as average values from the brain as a whole according to the method of KETY and SCHMIDT (1945) The induced changes were however rather small and the obtained values remained near the normal even if the changes were clearly significant

In normotension an impaired circulation is often treated with drugs which dilate the vessels by action direct on the vessel walls In general the cerebral vessels participate in the over all response to this type of drug (see SOKOLOFF 1959) The effects of drugs such as histamine and nitrites (DUMKE and SCHMIDT 1943) are very transitory and as they often cause an abrupt fall in blood pressure the cerebral blood flow is often even decreased On the contrary cerebral blood flow increased significantly after intravenous in

jection of papaverine (JAYNE *et al* 1952) Other investigations have furthermore reported the benefit of papaverine in the treatment of certain cerebral circulatory disturbances (RUSSEK and ZOHMAN 1948 MCCALL FINCH and TAYLOR 1951)

The purpose of the present study was to investigate the reaction of the cerebral vessels to some vasoactive drugs by use of the untraumatic method of LASSEN *et al* (1963) which allowed differentiation of the blood flow of the cerebral grey matter from the flow of the white matter (HÄGGENDAL, NILSSON and NORBÄCK 1965) This paper is concerned with the effects of Aramine noradrenaline and papaverine upon the vascular resistance and the blood flow of the cerebral grey matter in barbiturate anaesthetized dogs In order to evaluate the ability to autoregulation of the cerebral blood flow when the animals were subjected to intravenously infused pressor drugs the vascular response was studied either during conditions when systemic blood pressure was allowed to rise or when the blood pressure increase was prevented by withdrawal of blood In a few dogs similar procedures were performed under influence of induced slight hypoxia and/or hypercapnia The effect of papaverine was studied both alone and during the administration of Aramine

METHODS

The experiments were performed on a total of 14 unselected mongrel dogs Three experiments are not included in this study since they cannot reasonably be regarded as representative Two animals deteriorated due to large pulmonary atelectasis before the operative procedures were completed One experiment had to be discarded since the recorded activity after intracarotid injection of the radiokrypton was almost exclusively of extracerebral origin The results are thus based upon experiments in 11 animals weighing 8 to 14 kg in which 97 determinations of CBF were performed

The material has been divided into 2 groups according to the kind of pressor substance that was used metaraminol (Aramine MSD) or noradrenaline (Table I)

Group A (Table II) consists of 8 animals to which Aramine was given in varying doses 26 determinations of CBF were performed in these dogs under the influence of the drug at different levels of arterial blood pressure and in two dogs also during alterations of blood gas content Papaverine was given in a dose of 20—80 mg to 6 animals of this group and 7 determinations of CBF were done Four dogs were given Aramine and papaverine

TABLE I Subdivision of the material

	Number of animals	Number of CBF determination
Group A (Table II)		
Controls	8	25
Aramine	8	26
Papaverine	[illegible]	7
Aramine + papaverine	4	4
Group B (Table III)		
Control	3	15
Noradrenaline	3	17
Papaverine	2	3

simultaneously and 4 determinations of CBF were performed As controls 25 measurements of CBF were done in the 8 animals of this Group A at normotension and at lowered arterial blood pressure

Group B (Table III) is composed of 3 dogs on which 17 measurements of CBF were performed under the influence of noradrenaline in varying doses In addition papaverine was given to 2 of the dogs and 3 determinations of CBF were performed Control measurements of CBF were done in this group 15 times

The animals were anaesthetized with pentobarbital intravenously injected at an initial dose of 25—30 mg/kg body weight Supplementary doses of 3—5 mg/kg body weight were given at intervals of about two hours Generally the experiments lasted six to eight hours and thus the individual animals were given a total of 30—50 mg pentobarbital/kg body weight The rectal temperature was measured and maintained at approximately 38° C by means of an electric heating pad Clotting was prevented by means of intravenous administration of heparin The animals were curarized by administration of gallamine triethiodide (Flaxedil M & B) slowly injected intravenously in doses of 1 5—2 5 mg/kg body weight Additional doses of 0 5—1 0 mg/kg were given during the experiment The respiration was maintained via an endotracheal tube by means of a Starling Ideal pump so adjusted before the curarization that the tidal volume delivered barely suppressed the spontaneous respiration

Plastic cannulas were inserted into a femoral artery and vein in order to permit blood sampling pressure measurements and bleeding on the arterial side and administration of drugs and reinfusion of blood on the venous side

Arterial blood samples for blood gas analysis were taken not less than 30 minutes after the mechanical ventilation had started in order to ensure stable conditions and in most of the experiments a second control measurement was made 1—3 hours later. In those experiments where changes of ventilation were induced the levels of the arterial blood gases were considered to be stable within 30 minutes; this time period has earlier been demonstrated to be satisfactory (HÄGGENDAL *et al.* 1965).

Determinations of oxygen saturation were done according to the method of STENHAGEN (HOLMGREN and PERSSON 1959; BJURE and NILSSON 1963).

Measurements of the hemoglobin concentration were performed according to a spectrophotometric method of DRABKIN and AUSTIN (1935). For determination of arterial pH and carbon dioxide tension the micromethod of SIGGAARD ANDERSEN *et al.* (1960) was used.

The arterial blood pressure was recorded from the catheter in the femoral artery by means of an inductance transducer, an amplifier unit and a direct writing Mingograf (Elema). The mean pressures were obtained by electric integration. The horizontal plane through the entrance of the cannulated external carotid artery into the skull was taken as reference level.

Variations of arterial blood pressure were produced by controlled bleeding through the tube in the femoral arterial cannula into a reservoir where the blood was maintained at 38° C and from which it could be reinfused through the venous cannula.

The pressor substances were given intravenously by a mechanically driven syringe. Aramine was given in a concentration of 300—500 μg/ml and the infused amounts were from 1.5—40 μg/(kg body weight · min) and noradrenaline was given in concentrations of 10—50 μg/ml and the infused amounts were from 0.2—3 μg/(kg body weight · min). The infusions of the pressor substances started at least 5—10 minutes before measurements of CBF were done. Papaverine was given intravenously in doses of 1—10 mg/kg body weight.

Determinations of CBF were made according to a method which is a slight modification of that developed by LASSEN *et al.* (1963). Briefly summarized this method involves intra-arterial administration of radioactive krypton (Kr^{85}) to the tissue studied and external counting of the disappearance of the indicator. A thin polyethylene catheter was inserted via the lingual artery into the left external carotid artery and fixed with the very tip in proximity to the carotid bifurcation. This allowed the injected indicator to pass preferentially through the internal carotid artery. In a few dogs, however, the injections were done through a catheter in the vertebral artery. The catheter was then inserted via a sidebranch of the subclavian artery and advanced 5—8 cm up

into the left vertebral artery, the origin of which had been exposed. The catheters were filled with heparin saline solution and sluiced at intervals. The isotope Kr^{85} was delivered in a glass ampoule containing 1 curie (Radiochemical Centre, Amersham, England) and it was transferred into a glass syringe where it was equilibrated with about 40 ml saline (0.9 per cent NaCl). About 1—3 ml of this Kr^{85} solution was used for each measurement of CBF. Each injection was short, usually about 3 sec. For measurements of the γ radiation a scintillation detector with a 2 inch NaI crystal was used. The detector was connected to a ratemeter unit (Nukleoninkinstrument AB, Goteborg) from which the activity was recorded by a potentiometer writer (Varian recorder or Micrograph Kipp). Paper speed was in most cases set a 1 inch/1.5 min. The time constants of both ratemeter and recorders were 1 sec. The detector was placed over the left hemisphere and shielded by lead collimation so as to preclude as much as possible any measurement of extracerebral activity (i.e. activity accumulated in the neck, ears, nose etc.). The diameter of the collimator surrounding the detector was 5 cm.

The clearance curves thus obtained were recorded for 10—20 min. The activity was later plotted logarithmically against the time and the curves were resolved into two exponential phases (Fig. 1). There is strong evidence supporting the idea that the faster one of these components represents blood flow in the grey matter of the brain. The slow component is considered to be due to blood flow in other tissues to which the indicator had been administered, i.e. the white matter of the brain and extracerebral tissue (see LASSEN *et al.* 1963 and HÄGGENDAL *et al.* 1965).

This study is only concerned with the fast component, i.e. the blood flow in the grey matter. The reason for this selection of data presenting only the fast phase is that it was desirable to perform repeated measurements of CBF at short time intervals. Thus, the clearance curves could not be recorded long enough to permit resolution of a possible third component due to blood flow from slower perfused tissue (HÄGGENDAL *et al.* 1965). Despite careful collimation there was obviously some accumulated activity from extracerebral tissue which was measured by the detector. The accuracy of the half time of the second component was for this reason less certain. The influence on the first component was, however, negligible. When blood flow was calculated for grey and white matter respectively according to the formula (LASSEN *et al.* 1963)

$$CBF_{grey} \text{ in ml/(100 g} \cdot \text{min)} = 100 \times \frac{0.693 \cdot S_{grey}}{T\frac{1}{2}_{1st\ ph}}$$

and

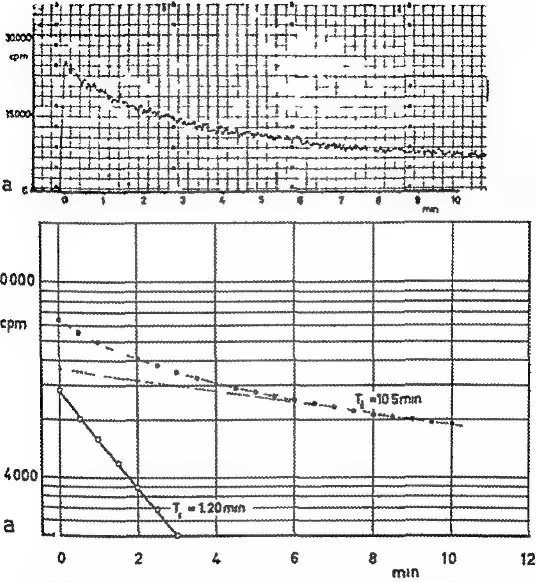

Fig 1a and b Comparison of two original curves and semilogarithmic replottings from the same dog during influence of a) Aramine in a dose of 6 5 μg/(kg min) (dog ~ d Table II) with a calculated blood flow in cerebral grey matter of 5. ml (100 g min)

$$\mathrm{CBF}_{\mathrm{white}} \text{ in ml/(100 g min)} = 100 \times \frac{0\,693 \quad S_{\mathrm{white}}}{T\frac{1}{2}\ _{1\ \mathrm{w\ phase}}}$$

there was a 5—10 times lower flow in the white matter This factor is presumably somewhat high because of the participation of extracerebral blood flow in the production of the second component

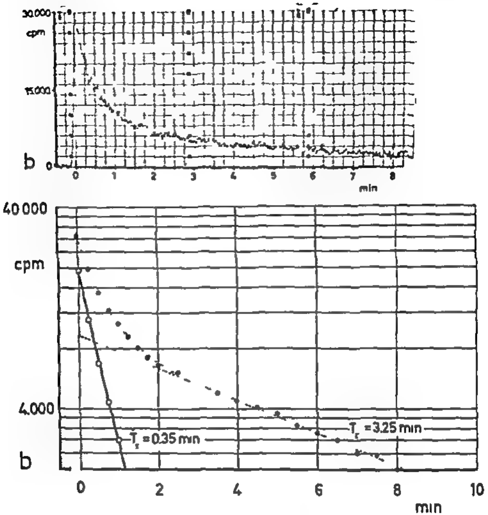

Fig 1 b

and b) papaverine in a dose of 4 mg/kg (dog " f Table II) with a corresponding blood flow of 187 ml/(100 g min)

The values used for the partition coefficients (S) between tissue and blood were 0 95 for the grey matter and 1 30 for the white matter used (GLASS and HARPER 1962 INGVAR and LASSEN 1962)

The flow resistance of the vessels in the cerebral grey matter (CVR) has been calculated from the mean arterial blood pressure and CBF_{grey} according to the formula

$$\text{CVR in mm Hg/(ml/100 g min)} = \frac{\text{mean art B P in mm Hg}}{\text{CBF}_{grey} \text{ in ml/(100 g min)}}$$

RESULTS

The results are presented according to the subdivision of the material into the two groups A and B (Table I) which was based upon the pressor substance used Aramine or noradrenaline respectively

Fig [illegible] (dog 5 Table II) illustrates the effects on arterial blood pressure cerebral blood flow and cerebrovascular resistance of Aramine infusions in varying amounts A total of ten CBF determinations were performed in two sequences separated by an interval of one hour

In (a) (b) and (c) CBF was repeatedly determined during control condition and was calculated to be about 120 ml/(100 g min) In (d) the arterial blood pressure was gradually raised by Aramine intravenously infused at a constant rate of [illegible] µg/(kg min) When mean blood pressure was stable at 200 mm Hg CBF was found to be 110 ml/(100 g min) To judge from this figure and from the results presented in Table II even moderate amounts of Aramine were able to reduce CBF despite the increased perfusion pressure In (e) when rate of infusion was doubled CBF was reduced to about 70 per cent of control in spite of a 50 per cent increase in arterial blood pressure During this period the calculated increase of CVR was 160 per cent of control An hour later when blood pressure had returned to the control value indicating that the effect of Aramine had been largely abolished a similar series of infusions were performed The blood pressure rise following the infusions was now however partially inhibited by withdrawal of blood and the mean arterial pressure was kept at approximately 200 mm Hg (g to j) The increase of CVR following the infusion of 3 µg/(kg min) was now even more pronounced (cf h and d) On the other hand an increase of the rate of infusion from 3 to 5 µg (kg min) caused hardly any further reduction of CBF (i)

At the end of the experiment Aramine was infused in a dose of 30 µg (kg min) Papaverine (2 [illegible] mg/kg) was injected during the Aramine infusion and flow resistance was markedly reduced (j) to below the control level

In accordance with the above results in the other animals in which blood pressure was raised by Aramine infusion (dogs 1—4 and dog 8) CBF was consistently reduced The most drastic reductions of CBF (about 50 per cent) were observed in animals with high initial flow values This reduction of flow was seen as response to infusions of Aramine at moderate rates Increase in the rate of infusion reduced flow values very little further The further increase of CVR seems entirely to be dependent on the level to which the arterial blood pressure was raised

During slight hypoxia and/or hypercapnia (dog 3 and 8 respectively) the effect of Aramine infusion was quantitatively of the same order

TABLE II Effects of Atropine and Papaverine on [illegible]

Exp dog	Bleed or transf	Atropine μg (kg min)	Papaverine mg kg	CBF	Mean art BP	CVR	O_2 cap	S_{aO_2}	P_{aCO_2}	pH
1a				83	1_5	1 5			40	7 41
b		1 8		6	35	3 4				
		1 r	1 0	10	_30					
d	1 0	1 5		71	130	1 8				
			1 0	III	,5	0 7				
[illegible]				11_	115	1 0	1,5	99	4	7 40
b		`		67	_00	3 _				
c				80	100	_ 4				
d	1 0	,		44	95	2 2	7	97	44	7 4
e			10 0	124	80	0				
f	— 0	5		66	100	1				
g		r	, 0	99	85	1 0				
[illegible]				146	140	1 0	_1 0	88	0(?)	7
b	0			77	95	1 1				
c		1 5		77	140	1 4				
d	+180			105	00	1 0				
	—100	1 5		99	_00	3				
f			3 0	110	180	1 0	3 0	97	39)	7 [illegible]
g	—1 0			89	00	1 0				
h		1 5		66	140	_ 1				
i	|100		3 0	_0	1 0	0				
4a				5?	1 0	_ 6	21 0	93	44	7 3
b	— 00			55	1 0	2				
c		3 0		57	_00	3 4				
f	— 0	_ 8		40	180	3				
e		10 0		[illegible]	2 5	4 0				
f	+100		, 0	150	_40	2 0				
g	—100	_ 5		101	140	1 4				
5				1 0	160	1 3		91	38	7 38
b				1 0	100	1 2				
c	— 0			116	160	1 4				
d		3 0		110	_00	1 3				
e		6 0		83	140	_ 9				
f				110	100	1 5		84	41	7 43
g	— 10	1 5		98	_00	_ 1				
h	— 30	3 0		77	100	2 5				
i	— 30	5 0		71	100	_ 6				
j		30 0	_ 0	104	1 0	1 _				

8₇										
l	−1 0	−0		118	110	0 4	1	98	4_	−3
c	− 0	1 0		−_	100	1 1				
l	−100	0		−_	100	1 4				
(	0	1 0		4	100	1 −				
t	1 3 0			−	100	1 3	1 1 8	81²)	²)	7 11
k	− 00			118	110	0 4				
1				111	50	0 1				
h	− 1₅			94	170	1 8			45	−40
	1 1₋			1	10	0 4				
l	−1 0	0		10	1 0	1 1				
(	− 50	1 0	0		155		19 4	93	10	−
f	1 1 0		4 0	k	140	1				
8₁				187	140	0 8				
h	− 0			74	140	1 4	3 1	11	4_	7 3
c	50			88	[illegible]	1 4				
d	+300			4	60	1 0				
(	735	1 0		74	140	1				
f	1 0	1 0		6	140					
k	_ 0	1 0		0	90	1 5				
h				1	90	1 1				
i	+400			18	140	1 4				
j	−200	10 0		140	1 10	1 4			4²)	−01
k				1	1 30	3				
			8 0	110	1 0	1 6				

¹) Ventilation with air mixture containing 3 per cent of carbon dioxide
²) Provoked hypoventilation

Exp dog = Experimental dog. The small letter after the figure indicates the chronological order of the measurements
Bleed or transf = Bleeding or transfusion in ml
$CBF_{g\ r}$ = The calculated blood flow of cerebral grey matter in ml/(100 g min)
Mean art B P = Mean arterial blood pressure in mm Hg
CVR = Cerebrovascular resistance in the grey matter calculated from mean art B P and $CBF_{g\ r}$ and expressed in mm Hg/(ml/100 g min)
O_2 cap = Oxygen capacity of the hemoglobin in the blood in vol per cent
S_{aO_2} = Arterial oxygen saturation in per cent
T_{aCO_2} — Arterial carbon dioxide tension in mm Hg
pH_a — Arterial pH

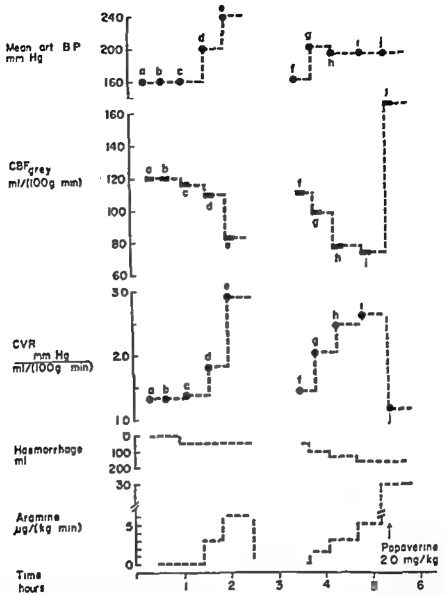

Fig [illegible] Effect of Aramine provoked hypertension on CBF and CVR (dog 5 Table II)
a b and c control measurements
d and e during infusion of Aramine the mean art BP and CVR increased resulting in a reduction of CBF these changes were more marked in (e) when the Aramine dose was doubled compared to (d)
f control measurement about one hour after the Aramine infusion was stopped Both blood pressure and CBF had returned to the initial control values
g h and i Aramine infusion in increasing doses with even more pronounced reduction of CBF compared with (d) and (e) Mean art B P was kept about 200 mm Hg by controlled bleeding
j when papaverine (2.0 mg/kg) was injected during Aramine infusion (30 μg/(kg min)) a marked decrease of CVR and increase of CBF were noted

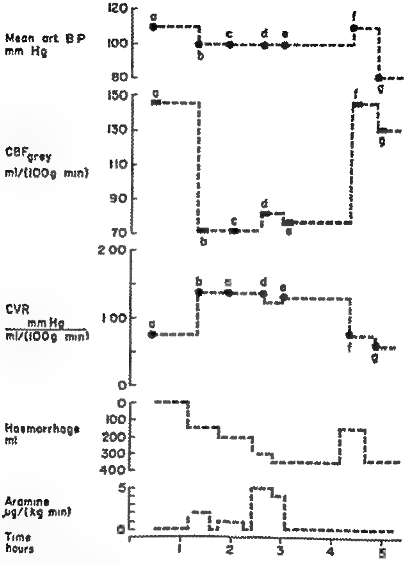

Fig . Effect of Aramine on CBF and CVR when mean art B P was kept at 100 mm Hg by withdrawal of blood (dog ■ Table II)

a control measurement

b—e during infusion of varying doses of Aramine there was a marked increase of CVR with accompanying reduction of CBF to about 50 per cent of the control value Note that increasing the rate of the Aramine infusion caused no further decrease in CBF

f and g when the Aramine infusion had been stopped for about one hour and blood reinfused both CVR and CBF had returned to about the initial values when blood pressure was lowered by bleeding autoregulation of flow was observed

Fig. 3 (dog 6 Table II) demonstrates the results of an experiment similar to that in Fig. 2. During Aramine infusion mean blood pressure was kept at 100 mm Hg by withdrawal of blood. Small doses of Aramine 1—2 μg/(kg min) caused a drastic decrease of CBF which could not be further reduced by increasing the rate of Aramine infusion 4—5 μg/(kg min). In this experiment CVP was increased by about 80 per cent. When the infusion was stopped and blood was reinfused into the animal CBF and CVP returned to their control values (f). — In order to control the condition of the cerebral vessels their autoregulatory ability was evaluated at the end of the experiment. Blood pressure was reduced from 110 to 80 mm Hg by blood withdrawal and CBF was found to be only insignificantly reduced (*cf.* f and g). Similar results were obtained in dogs 2, 3 and 8 (Table II) when measurements of CBF were performed during the control state and during Aramine infusion with the mean arterial blood pressure held at approximately the control level.

The results obtained from dog 8 (Table II) illustrate the effect of Aramine on CBF and CVP during *hypotension*. The autoregulatory capacity of the cerebral vessels was first demonstrated. By controlled bleeding of the animal a lowering of arterial blood pressure from 140 to 60 mm Hg was produced which caused CBF to decrease from 78 to 56 ml/(100 g min). CVP decreased as a sign of the dilatation of the cerebral vessels from 1.80 to 1.03 mm Hg/(ml/100 g min). When measurements were repeated at the corresponding blood pressures but during infusion of 9 μg Aramine/(kg min) CBF was reduced from 56 to 53 ml/(100 g min). The capacity for flow autoregulation was thus even more pronounced.

Hyperventilation of the animal abolished the autoregulation so that CVP was 1.43 mm Hg/(ml/100 g min) at blood pressure 140 mm Hg and 1.3? at pressure 190 mm Hg without Aramine infusion. When Aramine was given in a high dose 30 μg/(kg min) CVP increased to 3.?3 at blood pressure 100 mm Hg and the flow decreased to the same level as it had been earlier at the time when the animal was being normally ventilated (*cf.* 8j and g, Table II).

Effect of noradrenaline on CBF and CVR

The results are presented in Table III (Group B, dogs 9—11). As infusions of Aramine, infusions of noradrenaline were accompanied by a marked decrease of CBF and increase of CVP both during the induced hypertension and when blood pressure was kept at control level by bleeding. During hypotension the blood flow was maintained at the same reduced level as is illustrated by Fig. 4 (dog 11).

TABLE III Effects of noradrenaline (and papaverine) on cerebral haemodynamics (Group B)
(The abbreviations are explained in the legends of Table II)

Exp dog	Bleed or transf	Noradrenaline µg/(kg min)	Papaverine mg kg	CBF	Mean art B P	CVR	O_2 cap	S_{O_2}	P_{aCO_2}	pH
9a				95	1 0	1 9	11 7	94	30	7 43
b	−200	0 3		69	1 0	2				
c	−80	0 6		7	135	4				
d	200			95	1 0	1 6				
e	+ 80		2 0	1 0	15	1 9				
f	−1		0	110	100	0 9				
g	− 0	0 3		76	170	1 4				
h	− 60	0 8		68	100	1 4				
i	+ 0			88	110	1 3	10 1	91	31	7 3
j	−1 0			82	80	1 0				
10a				88	11	1 5				
b				92	1 0	1 3	10 0	96	3	36
		0 7		73	2 0	3 0				
d	−100	0 7		60	1 0	3				
e		0 7		5	14	7				
f	−100	0 7		80	110	1 3				
g	−100	0 7		60	80	1 1	18 1	91	37	7
h	+100			91	80	0 9				
i	200			31	140	1				
j	− 0			100	50	0 8	10 6	96	41	7 3
k	180	1 4		64	[illegible]	1 5				
l		3		69	1 0	1 8				
m	0	4		66	80	1				
11a				3	135					
b	0			10	100		10 8	91	31	7 40
c	−100			11	[illegible]	1 3				
d		4 0		30	1 0	1 0				
e				40	6	1 4				
f		1 0		6	100	3 0				
g	− 0	8		[illegible]	10	1 0	10 9	90	33	7 9
h	30	1 0		31	60	0				
i		2		31	80	0				
j	+300			7	130					
k	50			37	100	1 6				
l	+1 0		4 0	1 0	190	1 0				

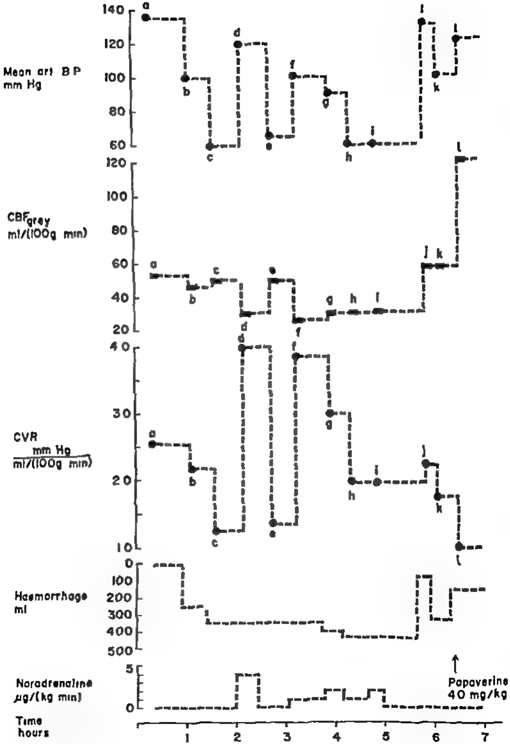

Fig 4 Effect of noradrenaline on flow autoregulation of the cerebral vessels (dog 11

At (a) in Fig 4 CBF was measured at the control blood pressure and at (b) and (c) blood pressure was reduced to 100 and 60 mm Hg respectively Despite this marked reduction of perfusion pressure CBF was almost constant demonstrating the autoregulatory capacity During this hypotension noradrenaline was infused and caused the arterial blood pressure to increase to about the control level (d) Despite this rise in perfusion pressure CBF decreased by about 40 per cent which means a more than threefold increase of flow resistance Control measurements were then performed (e) without influence of the pressor substance During (f) a minute amount of noradrenaline 1 μg/(kg min) was administered and CBF decreased to about the same value as in (d) which also occurred when the double amount of noradrenaline was infused in (g) During (h) and (i) the same procedure was repeated as in (f) and (g) but at the same low blood pressure as in (c) During this hypotensive state CBF was unchanged compared with (d) (f) and (g) thus indicating dilatation of the cerebral vessels as response to the decreased perfusion pressure This dilatation was independent of the rate of noradrenaline infusion (cf h and i) At the end of the experiment (j) and (k) measurements were performed under conditions similar to those in (a) and (b) Finally papaverine in a single dose of 4 mg/kg was injected (l) and CBF was drastically increased (see below)

Effect of intravenously injected papaverine on CBF and CVR

When papaverine was given with or without simultaneous infusions of pressor drug the result was a marked increase of CBF and decrease of CVR compared with control values When arterial pressure was high as a result of Aramine infusion CVR was higher compared with the situation when papaverine was injected without influence of pressor drug (see dog 1 Table

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Table III)

a—c control of autoregulation of CBF without drugs

d during influence of a moderate dose of noradrenaline blood pressure increased to about the control value in (a) but was accompanied with a more than threefold increase of CVR

e control measurement without noradrenaline during hypotension

f—g when noradrenaline was given in small amounts and blood pressure kept at about 100 mm Hg as in (b) CBF decreased to about the same value as in (d)

h—i a sequence similar to (f—g) but during hypotension as in (c) the cerebral vessels dilated due to the autoregulating mechanism and CBF was maintained at the same low level as in (d) and (f—g)

j—k repeated control measurements under conditions similar to (a) and (b) respectively

l a single dose of papaverine resulted in a marked decrease of CVR with corresponding increase of CBF

II) This higher CVR seems partly to be the result of an autoregulatory mechanism and partly to be due to vasoconstriction induced by the pressor substance. The existence of cerebral blood flow autoregulation under the influence of papaverine is illustrated by dog 9 (Table III) to which was given 2 mg papaverine/kg. CBF decreased from 120 to only 110 when blood pressure was reduced from 155 to 100 mm Hg by bleeding of 125 ml. CVR decreased from 1.3 to 0.9 and the control value of CVR at blood pressure 150 mm Hg was 1.8 mm Hg/(ml/100 g · min). That the Aramine provoked increase of CVR also existed when papaverine was given, although to a reduced extent, is illustrated by dog 7 (Table II). After previous infusion of Aramine, papaverine was injected and CBF increased from 55 when only Aramine was given, to 82 ml/(100 g · min) with a reduction of CVR from 2.5 to 1.7 mm Hg/(ml/100 g · min). When the Aramine infusion had been stopped for a while and only papaverine was given as a new intravenous injection CBF increased to 187 ml/(100 g · min) and CVR decreased to 0.8 mm Hg/(ml/100 g · min) at the same blood pressure.

Summary of the results

Aramine and noradrenaline, given as intravenous infusions in pressor doses, had qualitatively similar actions on the cerebral circulation in dogs although noradrenaline consistently seemed to have a more potent vasoconstrictor effect.

Both when the arterial blood pressure was raised by the pressor drugs and when the animals were kept at normotension by controlled bleeding there was a marked increase of the vascular resistance and decrease of blood flow in the cerebral grey matter compared with the corresponding control values in normotension. If the animals were hypotensive (60—80 mm Hg) due to haemorrhage and Aramine or noradrenaline was given, CBF was reduced to equally low level irrespectively of whether the blood pressure was allowed to rise or the hypotension was kept unchanged by further bleeding. Within a wide pressure range therefore, CBF was found to be rather constant at a low level during influence of these drugs. This implies that an eleva were hypotensive (60—80 mm Hg) due to haemorrhage was accompanied by a significant reduction of the blood flow in the cerebral grey matter. The cerebral vasoconstrictor effect of the pressor drugs were observed during slight hypoxia and/or hypercapnia. Papaverine intravenously injected was found to cause a marked vasodilatation of the cerebral vessels which also was obvious although less pronounced when Aramine was simultaneously influencing the vessels.

DISCUSSION

The main purpose of the present study was to investigate the vascular adjustments within the cerebral grey matter to the influence of certain vaso active substances: Aramine, noradrenaline and papaverine. Previous studies concerning these problems have dealt with average cerebral blood flow, i.e. a mean value of flow from both types of cerebral tissues, grey and white matter. In this study the Kr^{85} clearance method of LASSEN *et al.* (1963) was used in order to evaluate selectively the blood flow of the cerebral grey matter (see HAGGENDAL *et al.* 1965). As it was desirable to perform closely repeated measurements of CBF in the same animal under different experimental conditions, the clearance curves were recorded for only 10—15 minutes. This time was often too short to give the accurate slope of the second of the main components, especially when the CBF was low and the extracerebral activity high at the end of the experiment (HAGGENDAL *et al.* 1965). However, in control experiments it was observed that the influence of the substances used was much less on the second component than it was on the first.

Other methods of choice allowing selective evaluation of blood flow within the grey matter are the modification of the Kety technique according to HOMBURGER *et al.* (1946) or the Kr^{85} clearance method of LASSEN and INGVAR (1961). The former of these two methods has the disadvantage of being much more time consuming, thus reducing the possible number of observations. It also involves sampling of about 30 ml blood for each measurement of CBF. The γ recording technique was preferred above the β method because the former leaves the brain even more intact than the latter which requires exposed cortical surface. Furthermore, the assumed advantage of the β method for the actual problem of recording flow only from cortical tissue may probably only be valid when the cortex is thicker than 2—3 mm. The cortex of the brain in the dog is usually about 2 mm and it is possible that the recorded β curves contain a component from white matter due to penetration of Kr^{85} β particles through the cortex of the animal (HAGGENDAL *et al.* 1965).

As the main interest in the present study was directed towards the fast component of the clearance curves, it was considered to be of minor importance that the internal carotid artery was chosen in most of the dogs for the isotope injections. The greater supply from this artery to extracerebral tissue has only negligible effect on the resolution of the fast component of the clearance curves and the internal carotid artery is technically much easier to use than the vertebral artery.

The error induced by using the same partition coefficient for Kr^{85} between cerebral cortex and blood (0.95) without regard to variations in hematocrit (*cf.* LASSEN and MUNCK 1955) has been considered as negligible. The hemoglobin

concentration was determined at least twice in each animal at intervals of more than two hours any differences that occurred were so small that changes in the hematocrit dependent partition coefficient could not have been of importance for the accuracy of the calculated flow values

Concerning the assumed stable condition of the animals during the experiment, the level of anaesthesia varied in some degree as the pentobarbital was given at intervals but the variations were probably not too great was indicated by the cortical blood flow which showed only minor variations when it was controlled in similar states (HOMBURGER *et al* 1946)

Comments on the action of Aramine and noradrenaline on the vessels of the cerebral grey matter

In accordance with earlier studies Aramine and noradrenaline were found to increase the cerebrovascular resistance SENSENBACH MADISON and OCHS (1953) found that single doses of noradrenaline intramuscularly administered in amount varying from 700—1000 μg resulted in a mean increase (in 13 normal healthy males) of 64 per cent in CVR and in a 21 per cent reduction in CBF Mean arterial blood pressure rose 31 per cent KING SOKOLOFF and WECHSLER (1951) used continuous intravenous infusion of noradrenaline in 9 subjects to whom a mean dosage of about 500 μg was given They found a 37 per cent increase of CVR an 8 per cent reduction of CBF and a 29 per cent rise of mean arterial blood pressure MOYER MORRIS and SNYDER (1954) found a 50 per cent increase in CVP during noradrenaline infusion and 53 per cent when using Aramine CBF decreased 9 and 12 per cent respectively and mean arterial blood pressure increased about 30 per cent after both drugs

There are two recent studies which indicate that the constrictor effect of noradrenaline on cerebral vessels is weak BOHR GOULET and TAQUINI (1961) found no response from isolated smooth muscle preparations of cerebral vessels of dogs (200—300 μ in diameter) even to high concentration of catecholamines IKEDA *et al* (1963) reported a minimal decrease or even an increase of blood flow through the internal carotid artery in rabbits after intra arterial injection of noradrenaline

The present study strongly supports the opinion that Aramine and noradrenaline exert a vasoconstrictor effect also on cerebral vessels A usually pronounced reduction of CBF was a constant finding even under condition of a marked increase of perfusion pressure An increased or unchanged blood flow of the cerebral grey matter was never observed The reason that in the present study the vasoconstrictor effects seem to be even more pronounced

than in the earlier mentioned studies is probably that the vessels of the cerebral grey matter are more sensitive to these drugs than those of the white matter. The slow component of the clearance curves was not affected to the same extent as the fast one.

A marked constriction of the cerebral vessels seemed to occur even at rather low concentrations of the pressor drugs used. In most experiments the reduction of CBF was only slightly augmented when higher doses were given, indicating that the "therapeutic" doses which were used were great enough to give full response. During the infusion of pressor substances the cerebral blood flow was reduced to the same level whether blood pressure was allowed to rise or pressure elevation was prevented by withdrawal of blood. This constancy of decreased flow may be a result of the maintained ability of flow autoregulation which normally is operating (see e.g. HÄGGENDAL and JOHANSSON 1965). The ability of the vascular smooth muscle cells to change their tone when blood pressure is altered so as to maintain blood flow largely unchanged allows, of course, great variations of CVR also during the influence of pressor drugs. When blood pressure is kept constant, both Aramine and noradrenaline seem to increase the resistance by about 100 per cent. This pronounced vasoconstrictor effect can, however, be compared with the effect of hypocapnia which also produces a cerebral vasoconstriction of the same magnitude. It is also of interest to note that when the animals were hypoventilated or made hypercapnic by breathing a CO_2 mixture, the effect of Aramine was still unchanged, i.e. the cerebral vessels were constricted to about the same extent.

It may be of clinical importance to investigate the extent of vasoconstrictor influence of these pressor substances on the cerebral vessels in man during hypoventilation. In the clinical study performed by SENSENBACH *et al.* (1953) the pressor drugs caused no decrease in cerebral oxygen consumption, but this study was done in normally ventilated subjects. If the constrictor effect on the cerebral vessels is more potent than the dilator tendency of the prevailing hypoxia, it is possible that cerebral oxygen consumption has to decrease due to the decreased flow.

It may be assumed that the benefit of the pressor drugs with regard to the blood flow of the brain (FRANK *et al.* 1956) has been the result of a rise in blood pressure to a level where the autoregulation of the flow again occurred, thus resulting in an increase of blood flow despite increased vascular resistance. This may be the explanation of an earlier discussed differentiation of the effects of noradrenaline and Aramine on the cerebral circulation in the normal and the hypotensive states (SOKOLOFF 1959). In man during hypotension induced by ganglionic blocking agents, restoration of the blood pressure [illegible]

infusion of noradrenaline raises not only cerebrovascular resistance but also cerebral blood flow towards its normal level (MOYER, MORRIS and SNYDER 1954). In experimental hypovolemic shock in dogs, an increase of blood flow also accompanies the increased blood pressure after infusion of noradrenaline (FRANK *et al* 1956). SOKOLOFF (1959) explains this as "a difference in the mechanisms of the pressor response and its quantitative relationship to the change in cerebrovascular resistance". Normally, the vasoconstrictor effect of the drug on most vascular beds is antagonized to some extent by the response of the pressoreceptor reflexes to the rise in blood pressure. As the cerebral vessels do not seem to be under the influence of these reflexes, they may under these conditions become relatively more constricted than the over-all vascular bed. During the hypotension induced by the ganglionic blockade, however, the pressoreceptor reflexes are interrupted; the cerebral vessels may therefore be even less constricted than the peripheral vascular bed due to the chemical homeostatic mechanisms. During hypotension induced by bleeding instead of by ganglionic blockade, the elevation of cerebral blood flow by pressor doses of noradrenaline has been assumed to occur as a result not only of peripheral vasoconstriction but also of a rise in cardiac output which may be preferentially redistributed in favour of the brain and the heart (FRANK *et al* 1956, see also SOKOLOFF 1959).

This differentiated effects of the pressor drugs in the normal and hypotensive states may, however, be explained, to some extent at least, by the mechanism of cerebral blood flow autoregulation (LASSEN 1964, HAGGENDAL and JOHANSSON 1965). When the hypotension is so marked that the cerebral vessels cannot dilate further, the result will be a passive pressure-flow relationship; when the pressure is lowered beyond this level, the cerebral blood flow will decrease to very low values. During the pressor response induced by one of the pressor drugs the flow will increase first according to the passive pressure-flow curve and then stabilize on the autoregulated level; the net effect will then be a more or less pronounced increase of flow compared with that during hypotension. The cerebrovascular resistance will, however, indeed be higher than that of the normal vessels during normotension and without the influence of pressor drugs.

Comments on the effect of papaverine on the vessels of cerebral grey matter

Papaverine has been considered as an effective therapeutic drug in the treatment of many diseases with reduced cerebral blood flow, especially presumed vasospastic conditions. SHENKIN (1951) found in 4 subjects with cerebrovascular disease and reduced cerebral blood flow, after intravenously

administered papaverine a reduction of mean arterial blood pressure and cerebrovascular resistance but no significant increase of flow. JAYNE *et al.* (1952) reported from 18 subjects, most of them with cerebrovascular disease, a 13 per cent increase of cerebral blood flow after intramuscular administration of papaverine. There was also a 16 per cent reduction of CVR and a 13 per cent decrease of mean arterial blood pressure.

In accordance with these studies are the present results concerning the effect of papaverine on the vessels of cerebral grey matter in dogs, although the effects produced were more pronounced. The reduction of the cerebral blood flow resistance in several dogs was more than 50 per cent, and even when the cerebral vessels were under the influence of the given pressor drugs there was always a marked decrease of the cerebrovascular resistance and an increase of blood flow when papaverine was given. During hypercapnia or slight hypoxia this dilatation was also obvious. The effect of papaverine on the mean arterial blood pressure was a slight reduction of the pressure lasting a few minutes, whereas the dilating effect on the cerebral vessels of the grey matter was obvious for at least one hour. As was pointed out in the discussion on Aramine and noradrenaline, it would be of clinical interest to study the effects of papaverine, together with pressor drugs, on cerebral circulation in patients suffering from hypovolemic shock severe enough to have overcome the capacity for cerebral blood flow autoregulation.

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BY

JAN BRUNDIN

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CONTENTS

CHAPTER I

INTRODUCTION

The mammalian oviduct, or the Fallopian tube consists of three parts the isthmus connecting the oviduct to the uterus, the infundibulum ending in the periovarian space and the ampulla which forms the intermediary portion between the isthmus and the infundibulum

The main functions of the oviduct are to convey the ova from the ovary to the uterus and to secrete the constituents of the albuminous layer surrounding the ova In addition, the fertilization takes place in the ampulla (cf Young 1961)

The transfer of ova through the oviduct is assumed to be performed by a combination of muscular contractions in the wall of the organ and ciliary activity in the tubal epithelium The time required for the passage of ova from the fimbriated end of the oviduct to the uterus is about 3 days and is fairly constant for different mammals (Anderson 1927) including the rabbit (Assheton 1894) which was used as experimental animal throughout the investigations to be presented here

In their studies of the transport of ova through the rabbit oviduct Burdick and Pincus (1935) described and directed the attention to the fact that the ova were retained in the ampulla for 2—3 days before continuing down to the uterus (cf also Greenwald 1959 1961) A similar retention mechanism has later been observed in the cow oviduct (Black and Davis 1962) This retention of the ova can either be prolonged by injections of small doses of estrogen or shortened by larger doses of the same substance This has been shown in rabbits (Burdick and Pincus 1935 Pincus and Kirsch 1936 Black and Asdell 1959 Noyes, Adams and Walton 1959 Greenwald 1961 Harper 1964, 1965) mice (Burdick and Pincus 1935 Whitney and Burdick 1936, Burdick Whitney and Pincus 1937) and rats (Alden 1942) Administration of progesterone does not markedly accelerate the egg transport through the rabbit oviduct (Black and Asdell 1959, Greenwald 1961)

Several different explanations have been suggested for the physiological

mechanism underlying the retention of ova in the ampulla, *e g* temporary inactivity of the epithelial cilia, edema of the isthmic region, ad ovarian activity, constriction or inactivity of the isthmic circular muscles as well as tubal blocking by specific sphincteric muscle(s) in the isthmus

Some of these possibilities have been the subjects of investigations Thus, Borell, Nilsson and Westman (1957) reported an uninterrupted increased ab ovarian beating frequency of the epithelial cilia in the rabbit oviduct for a period of 14 days after ovulation Several attempts to demonstrate postovulatory edema in the rabbit tubo-uterine junction and the uterine part of the isthmus have been unsuccessful (Black and Asdell 1959) Photokymographic *in vitro* studies of circular muscle strips from the rabbit oviduct have revealed that the activity of these muscles in the isthmus was negligible from 24 to 96 hours *post coitum* as compared to the high activity of the circular muscles of the ampullary region and of the infundibulum during the same period (Black and Asdell 1959) From *in vivo* studies Westman (1926) concluded that only occasionally contraction waves could be seen to travel towards the ovarian end of the rabbit oviduct during the ovulatory period (cf Nakaso 1954) Simultaneously to a distension of the ampulla, constriction of the circular muscles of the isthmus has been observed after ovulation in the mouse (Burdick, Whitney and Emerson 1942) and in the rat, where the isthmo ampullary junction was constricted (Alden 1942) as well as in the rabbit (Black and Asdell 1958) In addition ligation of the fimbriated end of the rabbit oviduct has been reported to cause distension of the ampulla by accumulated epithelial secretion during the two or three days *post coitum* and to a lesser extent during anestrus, estrus and pregnancy (Hafez 1963) The distension was interpreted as evidence for lacking luminal tubo uterine communication during the greater part of the rabbit's sexual cycle Black and Davis (1962) have reported a similar distension of the cow ampulla for 72 hours after ovulation, while the entire isthmus appeared to be constricted during this time Stavorski and Hartman (1958) insufflated the rabbit Fallopian tube with CO_2 and concluded that the tubo uterine junction and the isthmus were responsible for the recorded enhanced resistance to insufflation while the resistance of the ampulla was negligible Moreover, a recent study of the spontaneous intraluminal pressure waves in the ampulla and in the isthmus indicated that a functional occlusion could occur between the isthmus and the ampulla in the rabbit (Brundin 1964 a)

Despite these accumulated indications of a sphincter like action of the isthmus in several mammalian species histological examinations of the rabbit oviduct (Greenwald 1961) and of the human Fallopian tube (Lisa,

Gioia and Rubin 1954) have failed to demonstrate any isolated sphincteric structure in the isthmus. In many mammals (Anderson 1927, 1928) the tubo-uterine junction *per se* offers high resistance to an applied intra-uterine overpressure, held to be due to the microanatomical construction of the tubo-uterine junction. However, a real valve like tubo-uterine junction has been reported only for the golden hamster (Bögli 1959).

The endocrine influence on the spontaneous motility of the oviduct (Westman 1926, Nakaso 1954, Sandberg et al. 1960, Greenwald 1963), on the size of the muscle fibers (Anopolsky 1928), as well as on the histological features and the cytochemistry of the epithelium (Fredricsson 1959 a, b) has been extensively studied. The neuronal influence on the oviduct has, however, not been elucidated. Except for the extensive work by Langley and Anderson (1894, 1895) showing *i. a.* that the hypogastric nerves convey all the sympathetic fibers to the rabbit oviduct, the only paper concerned with the neuronal influence on oviduct motility is a study published by Kok (1927 a) who observed that the rabbit oviduct contracted when he stimulated the sacral parasympathetic nerves (S_I—S_{III}), while stimulation of the sympathetic nerves (nn. hypogastrici) generally had a relaxing effect on the musculature of the same oviduct.

The aim of the present series of experiments was to investigate the distribution and function of the adrenergic nerves to the oviduct and specifically the eventual sphincteric function of the isthmus. The comparatively extensive information on the structure and function of the rabbit oviduct in addition to the suitable size of this organ in the rabbit constituted the main reasons for the use of this animal throughout the studies reported in this paper.

The presence of sympathetic innervation of the isthmus was studied by biochemical estimates of the noradrenaline and adrenaline content as well as by a histochemical technique. Studies on the function of the sympathetic nerves in the circular muscles of the rabbit Fallopian tube are reported. The method employed for the study of the closing power of the isthmus during perfusion of the oviduct with physiological saline solution at low flow rates is described in detail. The results obtained in rabbits are compared with some data on the adrenergic innervation of the female genital organs in man (Brundin and Wirsen 1964 b). Some possible clinical implications of the results are also discussed.

The following abbreviations have been used:

CA = catecholamine(s)	DA = dopamine
NA = noradrenaline	TUJ = tubo-uterine junction
A = adrenaline	IAJ = isthmo-ampullary junction

CHAPTER II

DISTRIBUTION OF NORADRENALINE AND ADRENALINE AND OF ADRENERGIC NERVE TERMINALS IN THE FALLOPIAN TUBE OF THE RABBIT

METHODS

Material

The experiments were performed at different times of the year (March, April and October) on albino rabbits (2 5—3 7 kg) selected at random among the available material which in its entirety was delivered from the same purveyor For details on numbers of animals see the various sections below Unless otherwise stated no pretreatment was given

Biochemical analysis

In a total of 122 Fallopian tubes the contents of NA and A were estimated by means of the method of Euler and Lishajko (1961) which is based on the fluorimetric technique of the trihydroxyindole method (Ehrlen 1948, Lund 1949) and permits separate determinations of NA and A after oxidative transformation to their respective lutines (Cohen and Goldenberg 1957, Price and Price 1957 Euler and Lishajko 1959) The fluorescence of the samples was further stabilized by ethylene diamine according to the method of Euler and Lishajko (1961) The same photofluorometer (Coleman, Model 12 C Coleman Instr Inc Maywood, Ill) was used through all series of estimations

After sacrificing the animals either by blow on the head or by air embolism the oviducts were immediately dissected out along with the mesosalpinx on each side Care was taken to separate the organs properly from the surrounding fat tissue and the serosa This operation was performed on ice In a few groups the organs were cut into one uterine half (isthmus) and one ovarian half (ampulla and infundibulum) (Zimmerman 1960, Greenwald 1961) before being transferred to a freezer (—20° C) The organs were never stored frozen for more than 48 h before extraction of their CA in 10 % trichloroacetic acid

In some animals hypogastric nerve section was carried out one week before estimation of the oviduct content of CA After anesthesia by iv injection of 45 mg/kg Pentobarbital sodium (Mebumalnatrium ©, ACO)

the inferior mesenteric ganglia were extirpated along with 1—2 cm of the hypogastric nerve(s) through an abdominal mid line incision The controls in this series were sham operated one week before the analysis The sham operation was performed identically except for the section of the nerves and the ganglion extirpations

Pooled samples had to be used because of the comparatively low NA content in single oviducts (Brundin 1964 c) Usually the oviducts from 8 animals were homogenized together and treated as one sample This technique was employed for the determinations of the CA content of the whole organs as well as of their isthmic and ampullo-infundibular portions respectively Pooled organs (from 10 animals) were also used for analysis of the CA content after pretreatment with reserpine (Serpasil ©, Ciba) After pretreatment with estradiol 17β (Pharmacia Ltd) and progesterone (Progynon ©, Schering AG) paired organs from 12 animals were used for the analyses

All NA values below 0 05 μg per sample were listed as zero The A fluorescence was very low or absent throughout the different series of estimations and has not been especially accounted for

The recovery was 78 to 85 % in these series of estimations Standard statistical calculations were used The deviations refer to standard errors of the means Student s t test was used for computing of statistical significance

Histochemical method

Histochemical investigations on the distribution of adrenergic nerve terminals in the rabbit oviduct were performed on organs from 8 animals, the normal distribution being studied in 5 and in the other 3 cases the distribution after extirpation of the inferior mesenteric ganglia along with 1—2 cm of the hypogastric nerve(s) this operation was performed, by means of the procedure described above, 7 days before the experiment

For the visualization of CA in the nerve terminals of the organs studied, the sensitive and specific method of Falck and Hillarp was used (cf Falck 1962, Falck Hillarp Thieme and Torp 1962, Corrodi and Hillarp 1963 1964) This method is based on the observation that primary CA (*e g* DA and NA) when enclosed in a dry protein layer are readily condensed to intensely fluorescent 3,4 dihydroisoquinolines via 1,2 3,4 tetrahydroisoquinolines under the influence of formaldehyde gas at a suitable temperature NA A and DA give rise to a green and serotonin to a yellow fluorescence In addition nonspecific autofluorescent material produces brownish fluorescence

The animals were killed by air embolism and immediately afterwards

small pieces from various parts of the oviducts were carefully dissected out and rapidly frozen in propene/propane (4:1) cooled with liquid nitrogen. The specimens were dried *in vacuo* for 5—6 days and subsequently treated with formaldehyde gas during 1 h at 80°C. The pieces were then embedded in paraffin, sectioned at 7—10 μ and mounted in Entellan (Merck) to which a few drops of xylene were added for deparaffination.

A Zeiss fluorescence microscope with a Zeiss 50 filter (2 mm) in the tube was used for the observations. The light source was an Osram HBO 200 high pressure mercury lamp with a Schott BG 12 filter (3—4 mm). The technical procedure was recently described in detail by Dahlström and Fuxe (1964), Norberg and Hamberger (1964) and Falck and Owman (1965).

Under the microscope some details of the histological features could be observed which are not visible in the photographic pictures because of the long time of exposure required for optimum visualization of the fluorescent structures.

RESULTS

BIOCHEMICAL ESTIMATES OF NORADRENALINE AND ADRENALINE IN RABBIT OVIDUCTS

Noradrenaline and adrenaline contents in the whole organ and in its different parts

The oviduct content of NA and A was studied in 30 rabbits, divided into 5 groups, each consisting of 6 animals. The results are listed in Table I.

Table I. Content of NA in 5 groups of pooled rabbit oviducts (12 organs in each group)

Group number	NA μg/g wet wt	NA μg/sample	NA Averaged μg/pair of oviducts
1	0.90	2.34	0.39
2	0.62	2.35	0.39
3	0.81	2.49	0.41
4	0.59	1.48	0.25
5	1.73	1.61	0.27
			Mean 0.34 ± 0.03

Table II NA in uterine (U) and ovarian (O) halves of pooled oviducts from 5 groups of rabbits (12 organs in each group)

	NA µg/g wet wt			NA µg/group of oviducts			NA averaged µg/pair of ovid		
Group number	U	O	Diff	U	O	Diff	U	O	Diff
1	1.72	0.37	1.35	1.75	0.59	1.16	0.29	0.09	0.20
2	1.98	0.11	1.87	2.12	0.23	1.89	0.35	0.04	0.31
3	2.19	0.34	1.85	1.91	0.59	1.32	0.32	0.09	0.21
4	1.80	≤0.05	≥1.75	1.48	≤0.05	≥1.43	0.25	≤0.05	≥0.20
5	3.77	0.81	2.96	1.09	0.52	0.57	0.18	0.08	0.10
Mean	2.29 ±1.19	0.34 ±0.42	1.96 ±0.84	1.67 ±0.18	0.40 ±0.11	1.27 ±0.21	0.28 ±0.03	0.07 ±0.01	0.20 ±0.03

P diff $0.01 > P > 0.001$

showing the values in µg/g wet weight of the tissue as well as the total amount of NA in each sample and for comparison the computed values per pair of organs The content of NA in the uterine and ovarian halves of the oviducts respectively in the same groups is shown in Table II from which it appears that the NA content of the uterine halves by far exceeds that of the ovarian halves The mean difference was calculated from the differences in each group of animals (pair tests)

Effect of reserpine

The effect of two different intravenous doses of reserpine on the oviduct NA content was tested in pooled organs from a total of 10 rabbits In one group two doses of 1 mg/kg injected (6 rabbits) 48 and 24 h respectively, and in another group one dose of 0.25 mg/kg injected (4 rabbits) 20 h before removal of the organs were found to deplete the NA stores in the oviducts below measurable values This is of significance for the functional studies reported below (chapter III)

Effect of hypogastric nerve section

Section of the hypogastric nerve(s) and extirpation of the inferior mesenteric ganglia were performed (cf above) on 2 groups of rabbits each consisting of 3 animals Simultaneously another group of 3 animals was sham operated After 7 days the oviducts were extirpated and sectioned into uterine and ovarian halves and the NA content in the groups of denervated animals compared to that in the sham-operated group The results obtained

Table III NA content of uterine (U) and ovarian (O) halves of rabbit oviducts 7 days after hypogastric denervation

Groups	μg/g wet wt U	μg/g wet wt O	μg/sample U	μg/sample O	μg/pair of oviducts (averaged) U +	O =	total
Denervated							
I (3 animals)	1.52	0.51	0.24	0.11	0.08	0.04	0.12
II (3 animals)	0.67	0.13	0.[illegible]2	0.05	0.07	0.02	0.09
Sham operated							
I (3 animals)	1.42	0.32	0.65	0.28	0.22	0.09	0.31

(Table III) show that after denervation the NA contents both of the isthmus and the ampulla were reduced to 1/3 of the corresponding amounts in the sham operated group. Likewise the mean total NA content in the oviducts of the denervated groups is about one third of that in the sham operated group. The mean total value of the sham operated animals agrees well with the mean value shown in Table I.

In these operated animals small retroperitoneal inflammations could be seen at the site of denervation, also along the mid line abdominal incision the peritoneum was irritated in all the rabbits.

Effect of estradiol and progesterone

A study was undertaken to determine whether estrogen and progesterone influenced the content of NA and A in the rabbit oviducts. For this purpose, daily i.m. doses of 50 μg estradiol 17 β were administered to 6 animals for a period of [illegible] days, 6 other animals received in addition to this dose, also 1 mg progesterone in daily i.m. injections during the 4 last days. After the usual procedure for removal of the organs the weights of the oviducts were determined and the NA and A contents of the total organs of each animal estimated.

The results obtained are listed in Table IV, from which it appears that the mean individual NA content amounted to 0.49 μg per pair of oviducts in the estradiol group and to 0.43 μg per pair in the estradiol progesterone treated group. The difference between these two groups is not statistically significant, nor do the mean values found differ significantly from those of the untreated animals (Table I).

Also in these cases the A content was too small to give a significant fluorescence.

Table IV The content of NA in paired oviducts of rabbits pretreated with estrogen and with estrogen + progesterone

	Estradiol 17 β	Estradiol 17 β + progesterone
Animal nr	NA µg/pair of oviducts	NA µg/pair of oviducts
1	0.24	0.38
2	0.44	0.50
3	0.66	0.38
4	0.58	0.43
5	0.53	0.44
6	0.50	0.42
Mean	0.49 ± 0.06	0.43 ± 0.02

Table V The weight of estrogen and estrogen progesterone pretreated paired oviduct of rabbits

I Estradiol 17 β		II Estradiol 17 β + progesterone		III Control group (12 untreated rabbits)	
Animal nr	g wet wt	Animal nr	g wet wt	Animal nr	g wet wt
1	1.565	1	0.958	1	0.342
				2	0.390
2	1.065	2	0.704	3	0.523
				4	0.349
3	1.799	3	0.707	5	0.496
				6	0.427
4	1.757	4	1.147	7	0.418
				8	0.311
5	1.325	5	0.612	9	0.412
				10	0.388
6	1.380	6	0.771	11	0.533
				12	0.546
Mean	1.48±0.11		0.82 ± 0.08		0.43 ± 0.02
	Difference I—II $0.01 > P > 0.001$		Difference II—III $0.001 > P$		

The pretreatments affected the weights of the oviducts (Table V), a large increase in weights being observed in the group treated exclusively with estrogen Judging from the values obtained in the estrogen progesterone treated group this increase seemed to be counteracted by additional

Table VI Values of oviduct NA content obtained in the series of experiments described in chapter II

Groups	NA µg/pair of oviducts
Untreated controls	0 34 ± 0 03
Reserpine treated animals	≤ 0 05
Denervated animals	0 12 — 0 09
Sham operated controls	0 31
Estrogen treated animals	0 49 ± 0 06
Estrogen progesterone treated animals	0 43 ± 0 02

injections of progesterone The difference in weights between the two groups is statistically significant $(0\,01>P>0\,001)$ Both groups exhibited significantly higher weights of the oviducts than those of the control group $(P<0\,001)$

The values of the NA content in the oviducts, obtained in the series of experiments described above and calculated in µg per pair of organs, are listed in Table VI

HISTOCHEMICAL OBSERVATIONS ON THE DISTRIBUTION OF ADRENERGIC NERVE TERMINALS IN RABBIT OVIDUCTS

Other observations (Brundin 1964 c) suggesting that the adrenergic nerve terminals were unevenly distributed along the Fallopian tube prompted a special histochemical study of their arrangement within the oviducts

Normal distribution

Fig 1 shows the location of a specifically fluorescent adrenergic ground plexus in the oviducts The adrenergic nerve fibers exhibiting this type of fluorescence increased in number from the ovarian end of the oviduct in the direction towards the uterus In the infundibulum and the ampulla the fluorescent nerve terminals occurred mainly in the perivascular areas and in a thin subserosal nerve plexus exhibiting the specific green to yellow green fluorescence A limited number of adrenergic nerve terminals could be seen among the smooth muscle cells in the walls of the ampulla and infundibulum In these parts of the oviducts very few nerve terminals were

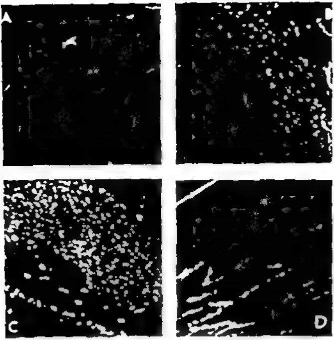

Fig 1 Micrographs showing adrenergic nerve distribution in rabbit oviduct

A Infundibulum near the ampulla Cross-section Few fluorescent terminals mainly at blood vessels 125 ×

B Isthmus Numerous fluorescent terminals in circular smooth muscle layer and around blood vessels Slightly oblique section 80 ×

C Tubo-uterine junction Abundant fluorescent terminals in circular muscle layer Slightly oblique section 80 ×

D Tubo-uterine junction Varicosities on fluorescent terminals in between circular muscle fibers Cross section 275 ×

observed in the mucosa On the other hand a thin strand of brown to yellow brown autofluorescent particles was regularly seen

The number of specifically fluorescent structures in the muscle layer was larger in the isthmus and in the region of the isthmo-ampullary junction than in the ampullary and infundibular parts of the organ The circular muscle layer was also more predominant from the isthmo-ampullary junction downwards growing gradually thicker towards the uterus In the parts of the organ next to the uterus the circular muscle layer constituted the main part of the cross section of the wall almost assuming the appearance of a sphincter A dense net of adrenergic nerve terminals occupying the outer half of this circular muscle mass was seen in all the isthmus preparations These fluorescent fibers which were richly equipped with terminal varicosities could be seen to intermingle with the circular muscle fibers of the isthmus, running almost in the same direction In the tubo-uterine

junction, as well as in the intramural parts, the adrenergic terminals were evenly spread through the entire circular muscle mass In the isthmic mucosa, however, only scarce nerve terminals were observed, while a thin layer of brown to yellow-brown autofluorescent particles was present the distribution of which resembled that in the ampulla and infundibulum Also in the longitudinal muscle layers relatively few fluorescent fibers were observed in the different oviduct preparations

Distribution after hypogastric nerve section

The distribution of the characteristic fluorescence seemed to be altered throughout the organs after hypogastric nerve section (cf Methods) In the infundibular and ampullary parts of the organs the number of visible fluorescent terminals of the subserosal plexus and of the thin muscular wall appeared to be reduced as compared to the controls In the isthmo ampullary junction the reduction was negligible, if at all present However, in the isthmic region between the isthmo-ampullary junction and the tubo uterine junction the reduction was more pronounced (cf Table III) The number of visible adrenergic nerves in the tubo uterine junction did not appear to be severely reduced by the nerve section

The reduction of the adrenergic innervation caused by this operation thus did not seem to be limited to any specific morphological structures of the rabbit oviduct though this reduction mostly affected the isthmus However, the visible perivascular fluorescent terminals also appeared to be slightly reduced and in one case no such terminals could be observed after the operation

DISCUSSION AND CONCLUSIONS

The fluorimetrically obtained CA values have been mainly listed as μg of CA per pair of oviducts in the present study The acceptable constancy of the CA values computed in μg/pair of oviducts is shown in Table I and II and constituted the main reason for reporting the data in this way In literature the common way of reporting the CA concentration in various organs has been to list the values as μg/weight of the examined tissue or as μg/total bodyweight of the experimental animal However, the weights of the oviducts showed a considerably wide individual variation in the control material (Table V), which would have caused the large variations of the NA values when computed as μg/g oviduct tissue (cf Table I) It has been reported previously that the weights of the female genital organs are influen

ced by the sex hormones Thus, administration of estrogens primarily causes water retention in the target organs (Astwood 1938 Holden 1939, Mueller 1957) hypertrophy of the tubal muscles (Wimpfheimer and Feresten 1939) and stimulation of the oviduct secretion whereas progesterone counteracts this secretory stimulation (Bishop 1956 a, b, Hafez 1963) For this reason varying degrees of water retention, due to the individual variations of endogenous incretion of these two hormones, may influence not only the weights of the oviducts (cf Table V) but also the NA values when computed as µg/g oviduct tissue In the present study no determinations of the oviducal dry weights were performed and therefore a correlation of the NA values to the dry weights was not possible

The NA containing nerve terminals in the muscular wall of the ampulla-infundibulum were almost exclusively found in the perivascular areas The NA values representing the ampulla infundibulum are likewise similar to those of other organs where only a perivascular adrenergic innervation is assumed to be present (Euler 1956) On the other hand, the abundance of adrenergic nerve terminals limited to the circular muscle layer of the isthmus corresponds to the relatively high NA concentrations in this part of the organ (cf Table II)

It is known from a large number of investigations that reserpine depletes the NA stores in different organs In the present studies the smallest dose used (0 25 mg/kg for 20 h) was found to deplete the NA stores of the oviducts This dose does not differ significantly from others previously found to deplete the NA content of other organs (cf Carlsson, Rosengren Bertler and Nilsson 1957)

The NA content of an organ can also be depleted merely by sectioning of the post ganglionic adrenergic fibers to the organ which causes the nerve fibers also within the organ to degenerate and to lose their transmitter (Cannon and Lissak 1939, Euler and Purkhold 1951 Goodall 1951) However, extirpation of the inferior mesenteric ganglia along with the proximal 1—2 cm of the hypogastric nerve fibers distal to these ganglia did not *cause a total depletion of the NA content of the oviducts but a reduction* to 1/3 of the control value (Table III) Provided all of the hypogastric fibers had been cut at the level of, and just below the inferior mesenteric ganglia the remaining third of the ordinary NA content indicates that a certain number of the adrenergic fibers to the oviducts were relayed to post ganglionic neurons in ganglia located peripherally to the site of denervation The presence of ganglia along the peripheral course of the hypogastric nerves has been previously described (Langley and Anderson 1894, 1895 Vanov and Vogt 1963) and some of these ganglia have been shown

to be adrenergic (Sjostrand 1962, Owman and Sjostrand 1965) The feeble reduction of fluorescent nerve structures in the tubo uterine and isthmo ampullary junctions after denervation indicates a larger number of peripheral synapses for the nerve fibers supplying these regions than for those ending in other parts of the isthmus and in the ampulla Denervation affected both the isthmus and the ampullary part (Table III) The pretreatment of the animals with estrogen and estrogen + progesterone did not induce any statistically significant changes in the CA content of the Fallopian tubes This is of importance for the functional studies in chapter III

It is known that the exact magnitudes of small amounts of A are difficult to evaluate in samples containing a comparatively high concentration of NA (Gunne 1963, Haggendal 1963) However, it may be concluded that the scarce appearance or absence of A indicates that only few chromaffin cells are present in the Fallopian tube of the rabbit (cf Euler and Purkhold 1951)

In the mammalian organism many smooth muscle sphincters are innervated by adrenergic nerves, *e g* the cardia (Norberg to be publ), certain sphincters of the urinary tract (Hamberger and Norberg 1965), as well as the internal sphincter of the anus This innervation should be reflected as an increase of the NA concentration in the sphincter region (Euler 1956) This should also be the case in the rabbit organs as NA has been shown to be the adrenergic transmitter substance in this species as in other mammals (Mirkin and Bonnycastle 1954)

Thus, the results of the present biochemical, histological and histochemical studies congruently indicate that the muscular and neuronal arrangements in the rabbit oviduct isthmus is such that this entire part of the organ may possess a constrictive, sphincteric function, regulated by adrenergic nerves

In the next part of the study (chapter III) this suggestion has been investigated from a functional point of view

CHAPTER III

FUNCTIONAL STUDIES ON THE ADRENERGIC INNERVATION OF THE CIRCULAR MUSCLES OF THE RABBIT OVIDUCT

The rich adrenergic innervation found to be predominantly localized to the circular musculature of the isthmus (chapt II) indicated a specific function of this part of the rabbit oviduct, *viz* that the isthmus might possess a sphincteric action modifying the resistance to flow through this part of the oviduct A series of experiments was therefore carried out to study the state of contraction of the isthmic circular muscles following various experimental procedures including electrical stimulation of the sympathetic nerve fibers to the oviduct The influence of certain substances on the functional state of this musculature was also studied

Changes in the state of contraction of the isthmus have previously been recorded by several methods A large number of *in vitro* methods have been described and applied for studies both of the entire organ (Blair 1922 Kok 1927 b, Mikulicz Radecki 1930, Freund Wiederman and Saphier 1963) and of various parts of its longitudinal (Sandberg et al 1960) as well as its circular musculature (Kok 1929, Black and Asdell 1959 Hawkins 1964 see also chapt I) On the other hand, no useful method seems to have been described for studies of the isolated oviduct with intact nervous supply In consideration of the topography of the nerves supplying the oviduct such an isolated preparation would be difficult to prepare

For studies of the oviduct musculature *in vivo*, Mikulicz Radecki (1926) described a method by which the tension of the circular muscles was recorded via small hooks transversely attached to the walls of the oviduct in rabbits Indirect methods of recording the state of contraction of the Fallopian tube have also been described Rubin (1920) introduced a so called uterine kymoinsufflation method by which a gas is allowed to pass from the uterine cervix through the uterus and the oviducts into the abdominal cavity The pressure required to force the gas through the Fallopian tubes was used as an index of the patency of the oviducts (Westman 1929, 1942 1957 Morse and Rubin 1937 Geist, Salmon and Mintz 1938) This method has also been used with modifications by Stavorski and Hartman (1958) in

e Horton, Main and Thompson (1963) employed an indirect meth- ised on the same principle but instead of a gas they infused a fluid ode s solution) through the uterine end of the oviduct while recording erfusion pressure as an index of the resistance to flow

r the recording of changes in the state of contraction of the isthmus g electrical stimulation of its sympathetic nerves a technique had to be oyed which produced as little trauma as possible to the nerve supply, ll as to the musculature An indirect method similar to that of Horton (1963) was therefore adopted Their use of fluid instead of compressible ould be expected to provide a higher degree of accuracy in the record

METHODS

btain an index of the state of contraction of the Fallopian tube the ure was recorded in a catheter, the tip of which was inserted into rent parts of the lumen of the oviduct and which was perfused with % NaCl solution at slow rate from a constant volume pump The per e was allowed to drain freely out of the opposite end of the oviduct A ire kept the catheter in position and prevented leakage between the ter and the wall of the Fallopian tube

s known that the resistance to flow through a tube is inversely propor l to the square of its cross sectional area (Poisseuille 1846) This law ld provided the length of the perfused tube and the viscosity of the isate remain unchanged during the perfusion In the present study a ening of the oviduct due to contraction of the comparatively thin · longitudinal muscle could possibly have caused a decrease, while a ation might have produced an increase, in the resistance to flow How- close observation of the Fallopian tube did not reveal any variations e length of the oviduct during experiments with intermittent electrical ılation of the adrenergic nerves It therefore seems unlikely that tions of the length of the organ should have been large enough to in ice the recorded resistance to flow Accordingly the possible changes in nal diameter which theoretically might be produced by a change in th of the organ may be considered negligible

ome variation in the viscosity of the perfusate may possibly occur during perfusion by the addition of secretory products to the perfusate from the ielium The maximum rate of secretion from the epithelium of the e rabbit oviduct during estrus and the first three days after mating

has been estimated to 0.032—0.057 ml/hour (Bishop 1956a, Clewe and Mastroianni 1960, Hafez 1963). The volume of this spontaneous secretion would increase the amount of flow through the oviduct by 3—6 % during perfusion at a rate of 16 μl/min. The amount of secretion during an experiment could possibly vary during the stimulation of the hypogastric nerves. However, the number of adrenergic nerve endings found in the mucosa of the rabbit oviduct is quite small (cf. chapt. II). Increased cholinergic activity would perhaps be more likely to modify the secretion since injection of pilocarpine is known to increase the rate of secretion and the amount of solids in the secretion (Bishop 1956a). In the experiments to be reported here, however, cholinergic influence may be assumed to have been effectively blocked by pretreatment with atropine (20 mg/kg) used in all the experiments. Variations in the rate of epithelial secretion or its viscosity might therefore also be disregarded as a source of error for the recording of the resistance to flow.

Thus, it seems justified to consider changes in the internal diameter of the oviducts as being the major factor in determining the recorded variations in perfusion pressure. The internal diameter is determined by the tone of the circular muscles of the organ. Electrical stimulation of the extensive net of adrenergic nerve fibers ending especially in this circular muscle of the isthmus would be expected to influence the tone of this muscle layer and hence the internal diameter of the isthmic part of the oviduct.

Material

The material consisted of albino rabbits (2.5—3.4 kg). Successful experiments were carried out on a total of 46 rabbits. For details on numbers of animals and pretreatment, see the various sections below.

Preparation

The rabbits were anesthetized with pentobarbital sodium (35—45 mg/kg i.v.). An i.m. dose of atropine (atropine sulphate), 20 mg/kg was ordinarily given to facilitate anesthesia. During the anesthetization heparine (4000 IU) was given to prevent clotting. The animals were placed in a supine position on a heated operating table and tracheotomized. One femoral artery was cannulated for the recording of systemic arterial blood pressure. Through a low abdominal mid line incision one of the uterine horns and the corresponding oviduct were identified and inspected to be macroscopically normal. A silk suture was loosely threaded around the oviduct at the level where the tip of the catheter was to be placed. Care was taken to cause as little damage

as possible to the vascular supply during this procedure The slightly everted tip of the perfusion catheter was inserted either from the isthmic end of the oviduct, via a blunt incision in the uterus 2—3 cm from the tubo-uterine junction, or through the abdominal ostium of the oviduct The catheter was carefully passed under the silk suture which was then gently tied around the catheter below its everted tip The intestines were kept aside in order not to interfere with the perfusion resistance of the oviduct by compression Before further preparation the spontaneous resistance of the oviduct was measured by repeated perfusion tests (see below) Through an incision in the dorsal peritoneum just below the branching of the inferior mesenteric artery, the hypogastric nerves and the inferior mesenteric ganglia were identified The retroperitoneal fat was sometimes abundant Variable amounts of fat were therefore left around the nerves Stimulation electrodes were placed around the hypogastric nerve bundle(s) just below the inferior mesenteric ganglia and proximal to the division of the nerve fibers into thinner filaments The experimental arrangement during perfusion from the uterine end of the oviduct is schematically shown in Fig 2

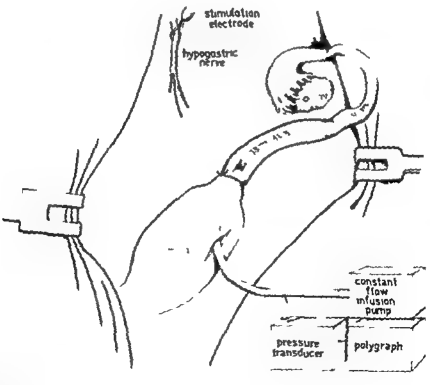

Fig 2 Sketch of preparation used for the functional studies Left uterine horn and adnexa exposed

Method of recording

The polyethylene catheter (Clay Adams Inc.) used for the perfusion of the oviduct was either PE 10 (0.28 mm inner — 0.61 mm outer diameter) or PE 50 (0.58 mm inner — 0.97 mm outer diameter). The catheter was filled with 0.9 % NaCl solution and attached to a motor driven syringe delivering a volume of either 16 or 28 (± 2) μl/min. From a T tube on the catheter the perfusion pressure was recorded by means of a strain gauge transducer (Statham P23 AC) with a volume displacement of 0.0083 mm^3/mm Hg. The same type of strain gauge transducer was also used for recording of the systemic arterial blood pressure. For the electrical stimulation of the hypogastric nerve fibers a square pulse stimulator (Grass S4 G) was used. Two platinum stimulation electrodes 2.5 mm apart were connected to the stimulator via a stimulus isolation unit (Grass SIU 4B). A laboratory timer (Perman and Persson 1962) was sometimes used for automatic monitoring of the duration of, and the intervals between the stimulation periods. All recordings were made on a polygraph (Grass Model 5 P1) equipped with DC coupled preamplifiers (Grass 5 P1, Grass Instr. Co., Quincy, Mass.). The damping of the electronic equipment was kept constant (frequencies over 15 cycles/sec were filtered out). By the use of the same length and kind of catheter, the same type of fittings etc. in all experiments the mechanical damping of the recording system was also kept constant during the different series of experiments. The perfusion was usually performed from the uterine end of the isthmus but also from various levels of the oviduct further up in the ovarian direction or from the ovarian end of the oviduct in the uterine direction.

Check of preparation

The sensitivity of the recording unit was calibrated hydrostatically against saline columns and also electrically against a circuit in the preamplifier corresponding to + 100 mm Hg. Before and after each experiment the pressure resulting from perfusion of the catheter itself with free tip was checked not to exceed 2 mm Hg at a flow rate of 28 μl/min. Likewise the catheter system was infused at the beginning and the end of each experiment at the same flow rate with the tip of the catheter occluded, using the same sensitivity of the amplifiers and the same speed of the recording paper as during the actual experiments. This infusion was discontinued at a pressure of 150—200 mm Hg and if the pressure subsequently did not decrease more than 5 mm Hg in 30 sec. the tightness of the perfusion system was considered acceptable. The rate of rise in the pressure recording system

during infusion with the catheter tip clamped was later used for the interpretation of the pressure curves obtained during the actual experiments. During an experiment, the occurrence of a rate of pressure rise, identical to that obtained with the catheter tip clamped would indicate that the Fallopian tube was completely occluded (see below). If this rate of rise was present already at the start of an infusion period it would indicate that the closure of the oviduct was located close to the tip of the catheter.

During the preparation the position of the catheter tip could be checked with direct observation or with transillumination. An inadvertent, extraoviducal position of the catheter tip could also be detected during the perfusion tests prior to the actual experiment. The stabilization of the perfusion pressure, normally established after a few minutes of perfusion was not obtained if the catheter tip was located outside the oviduct. When, for control, the tip was deliberately forced through the wall of the oviduct and placed between the two blades of the mesosalpinx, the perfusion pressure showed a successive increase which often amounted to more than 300 mm Hg already during the second or third perfusion test period of 2—3 min. An aberrant position of the catheter tip as well as subserosal rupture of the oviduct could also be detected as a swelling of the mesosalpinx after infusion for some time. In successful preparations the perfused solution could be seen to leave the free end of the oviduct during the experiment. In a few cases bleeding at the place of ligation of the catheter tip or cyanosis of the oviduct was observed. These preparations were discarded.

Provided the test perfusions showed satisfactory results and the subsequent nerve preparation had been successful, the effect of hypogastric nerve stimulation was tested during perfusion. If the electrical stimulation increased the resistance to flow through the oviduct the preparation was further used for the actual experiments. The test with electrical stimulation was not performed after pretreatment with *e g* reserpine. When it had been established that the preparation was satisfactory from a technical point of view the hypogastric nerve fibers were sectioned proximal to the stimulation electrodes.

In control experiments with occluded tip of the perfusion catheter, inserted through the uterus past half of the length of the isthmus, it was found that the contractile tissues then surrounding the distal portion of the catheter did not influence the perfusion pressure, even during strong electrical stimulation of the hypogastric nerves causing visible contractions of the uterus and the oviduct isthmus.

The experiment was discontinued after 3—4 hours even if the animal was in a good general condition as judged by the breathing and systemic ar-

terial blood pressure. An impaired general condition of the animal already before this time caused earlier discontinuation of the experiment. Immediately after each experiment, a vaginal smear was taken and instantly fixed (Spray-Cyte ©, Clay Adams Inc.) for estimation of the endocrine status of the animal.

Interpretation of the perfusion pressure curves

For the estimation of the results obtained it was necessary to know the rate of rise described by the pressure curve, obtained during perfusion of the catheter system versus the clamped tip of the perfusion catheter used (see above). The rates of rise of the clamped tip curves were therefore recorded before and after each experiment. During this procedure the same sensitivities, paper speeds and rates of infusion were used as during the actual experiment. Provided these curves showed identical rates of rise before and after the experiment, the results obtained were considered useful for further treatment. The end point of the yield pressure necessary to induce perfusion of the oviduct was measured by the same technique throughout the different series of experiments and was considered to be reached at that moment when the rate of rise of the pressure curve became less than that of the corresponding clamped tip curve. This pressure will be referred to as "the opening pressure". The term "spontaneous opening pressure" refers to the "opening pressure" obtained when the oviducts were perfused without nerve stimulation. The term "perfusion pressure" refers to the mean level of perfusion pressure around which the spontaneous pressure variations could be seen to fluctuate. The term "detectable response" refers to an increase of the mean perfusion pressure of ≥ 10 mm Hg, regardless of the rate of rise. The term "occlusive response" refers to a rate of rise of the perfusion pressure identical to that of the clamped tip curve, *e.g.* during hypogastric nerve stimulation.

Special methods

In some experiments castrated animals were used. The operation was done one month before the experiment under pentobarbital anesthesia. The ovaries were completely removed through an abdominal mid line incision without ligation of the mesovaria. Ligation had previously been found to cause comparatively large postoperative inflammation around the fimbriated end of the oviducts (Brundin 1964 b), and this complication was considerably reduced when the ligation was omitted. In another series of experiments the animals had been subjected to sectioning of the hypogastric

nerves one week prior to the experiment This operation was done in the same way as described in chapter II

When the perfusion of the oviduct was undertaken from the ovarian end of the organ, a catheter (PE 190) was placed in the uterine lumen close to the tubo-uterine junction for draining purposes

RESULTS

General observations on estrous and anestrous rabbits

An initial increase of the perfusion pressure was generally recorded when either the ampulla or the isthmus of the rabbit oviduct was perfused This pressure increase exhibited the same rate of rise as when the perfusion system was infused against the occluded tip of the perfusion catheter (Fig 3) When the opening pressure had been reached, an additional small increase of the perfusion pressure was recorded in most animals, corresponding roughly to half the amplitude of the spontaneous motility The mean perfusion pressure remained fairly constant after this period The opening pressure as well as the level of the mean perfusion pressure were found to vary with the part of the oviduct perfused The highest opening pressure and mean perfusion pressure were recorded with the tip of the catheter in a position 0 5 cm from the TUJ during perfusion in ovarian direction In

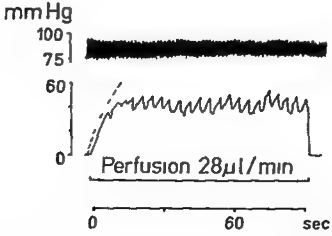

Fig 3 Pressure recording (bottom tracing) during perfusion in ovarian direction Opening pressure about 35 mm Hg and mean perfusion pressure about 40 mm Hg Catheter tip in isthmus 0 5 cm from TUJ Dotted line occlusive rise in perfusion pressure Top tracing systemic arterial blood pressure Smear atrophy

these cases a mean opening pressure of 31 5 ± 1 3 mm Hg was recorded during 78 perfusion tests upon 26 non pregnant rabbits (17 anestrous and 9 estrous rabbits) In the two first test perfusions of a series on the same preparation a higher opening pressure (45 9 ± 4 7 mm Hg) was found but decreased afterwards to a value which remained fairly constant during the following 15—25 test perfusions No decrease of the perfusion resistance could be observed in 5 cases after transsection of the ampulla just distal to the IAJ The smaller the perfused part of the isthmus the lower were the recorded opening pressure and mean perfusion pressure These observations indicated that the isthmus was responsible for the greater part of the opening pressure and the resistance to flow through the entire oviduct If only the ampulla was perfused after introducing the tip of the perfusion catheter to the IAJ or through the abdominal ostium a lower value was obtained for the opening pressure (11 2 ± 5 1 mm Hg) during 11 estimations on 5 animals If the entire oviduct was perfused from the abdominal ostium the same and comparatively low opening pressure was recorded as when the ampulla was perfused alone In these cases the perfusion pressure gradually increased to a level of about 30—40 mm Hg during the first 1—2 min of perfusion the rate of rise being less than during the clamped tip tests The course of the pressure curve indicated that the level of the perfusion pressure was determined by the resistance to flow in the isthmus when the ampulla had become distended and a subsequent flow through the isthmus had started Thus the perfusion pressure finally reached approximately the same level as when the oviduct was perfused from the proximal part of the isthmus in ovarian direction

The importance of the shape of the perfusion pressure curve for the interpretation of the pressure curves has been discussed above (see Methods) When the pressure increase showed a smaller rate of rise than that observed during the clamped tip test the passive and/or active occlusive power of the oviduct apparently had been overcome and a certain degree of flow through the isthmus had occurred By placing small air bubbles in the perfusion catheter, it could be established that when the perfusion curve showed a smaller rate of rise than the clamped tip curve the air bubbles were observed to move in the direction of the perfusion

When the mean perfusion pressure after the initial occlusive rate of rise exhibited a horizontal course the mean resistance to flow through the isthmus could be considered fairly constant It is apparent from Fig 3 that even if thus stabilized mean perfusion pressure as a whole described a horizontal course the spontaneous muscular activity of the oviduct caused the perfusion pressure curve to oscillate around the mean pressure Air bubbles,

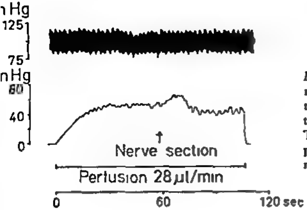

Fig 4 Section of hypogastric nerve during oviduct perfusion in ovarian direction Slight fall in perfusion pressure Catheter tip in isthmus 0 5 cm from TUJ Top tracing systemic arterial blood pressure Dotted line occlusive pressure rise Smear estrus

placed in the perfusion catheter for control, could be seen to move step wise in the direction of the perfusion when spontaneous activity occurred Simultaneously to the falling slope of the curve the bubbles moved relatively rapidly while they were retarded and often stopped when the spontaneous contractions caused a rise in perfusion pressure It was observed that the perfusion pressure curve during these spontaneous contractions often exhibited an occlusive rate of rise The spontaneous motility of the oviducts showed considerable variations of both amplitude and frequency from one experiment to another as well as during a single experiment, and as a result definite conclusions about this activity have been omitted

The distension of the organ during the perfusion tests was quite small and did not influence the systemic arterial blood pressure Neither was any consistent difference observed between the estrous and the anestrous rabbits concerning the shape of the perfusion pressure curves or the opening pressures recorded

Effect of hypogastric nerve section

Acute denervation In an early stage of each experiment the hypogastric nerves were cut proximal to the stimulation electrodes No statistically significant decrease of the opening pressure of the isthmus was observed during the perfusion tests immediately after this sectioning of the nerves When the nerve section was undertaken during perfusion, the mean perfusion pressure decreased by 5—10 mm Hg in 2 cases out of 5 after a temporary rise (Fig 4)

Chronic denervation The oviducts of five rabbits were studied 1 week after sectioning of the hypogastric nerves (see Methods) The opening pressure of the isthmus was 29 1 ± 3 5 mm Hg in this material *i e* similar to that found in preparations with intact nerve supply It may be pointed out in

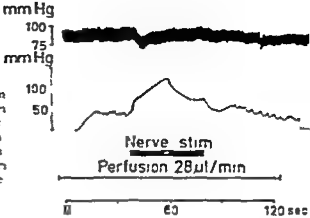

Fig 5 Hypogastric nerve stimulation (17.5 p s) during oviduct perfusion in ovarian direction. Occlusive rise in perfusion pressure at beginning of nerve stimulation. Dotted line occlusive pressure rise. Catheter tip in isthmus 0.5 cm from TUJ. Top tracing systemic arterial blood pressure. Smear a raphy

this connection that the NA content of the isthmus after the denervation was still high enough to allow adrenergic constriction of the isthmus (cf chapter II) This part of the rabbit oviduct had also exhibited a high sensitivity to NA in earlier *in vitro* experiments (Brundin 1964 b) The spontaneous motility was not changed by denervation In this series of experiments as well as in all others to be presented here the opening pressure was not changed when the rate of perfusion was increased from 16 to 28 µl/min

Effect of electrical nerve stimulation

During electrical stimulation of the hypogastric nerves the perfusion pressure was regularly affected (Fig 5) The frequency of the square wave pulses used was 15—20 p/sec the duration 1.5—2.0 msec and the stimulus strength set to 20—50 V on the stimulator As a rule the electrical nerve stimulation caused the perfusion pressure to increase by the same rate of rise as during the clamped tip test during isthmus perfusion In certain cases (*e g* Fig 5) an increase of the pressure curve with a brief initial steeper rate of rise than that in the clamped tip test was recorded This steeper pressure rise was probably due to retrograde expulsion of the small isthmic content, present in the lumen of the isthmus as perfusion through the organ had once started During the electrical stimulation a mean opening pressure of 79.5 ± 2.4 mm Hg was recorded in 59 repeated tests of 12 oviducts perfused in ovarian direction and with the tip of the catheter situated in the isthmus 0.5 cm from the TUJ A pressure curve exhibiting an occlusive rate of rise with readable opening pressure was never observed during nerve stimulation during perfusion of the ampullary half of the organ from the IAJ Merely a slight and gradual increase of the mean perfusion pres-

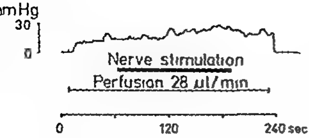

Fig 6 Hypogastric nerve stimulation (20 p/s) during perfusion of ampulla in ovarian direction. No occlusive response only a slightly increasing perfusion pressure Catheter inserted through isthmic lumen to IAJ Dotted line occlusive pressure *i e* Smear estrus

sure was observed in these latter cases during stimulation of the hypogastric nerves The spontaneous motility of the ampullary half was maintained during the nerve stimulation used and caused certain difficulties in estimating the increase in the mean perfusion pressure (Fig 6) The stimulation response from the ampulla disappeared after transsection of the organ in the IAJ

The experiments with electrical stimulation thus showed that the spontaneous opening pressure of the entire oviduct was determined by the state of contraction of the isthmus and that nerve stimulation caused only slight variations in resistance to flow during perfusion of the ampullary half of the organ The attention was therefore more or less exclusively directed to the isthmic part of the oviduct When not otherwise stated, the observations reported below will therefore refer to perfusion of the organs in ovarian direction with the catheter tip located in the isthmus 0.5 cm from the TUJ

With the perfusion rates used (16 and 28 µl/min) the full contraction response to nerve stimulation took some time to develop (Fig 7) The use of the higher or lower infusion rate was not observed to influence this latency However, the duration of the applied electrical stimulation affected the pressure response If the stimulation was maintained for more than 25—30 sec, the level of perfusion pressure decreased in spite of the continued stimulation (see for instance Fig 5) This 'escape' of the perfusion pressure was consistently more rapid in its initial phase When the stimulation was discontinued, the pressure curve declined to a value which in the greater part of the experiments was lower than that prior to nerve stimulation The post stimulatory inhibition not only decreased the mean perfusion pressure but also the spontaneous motility, normally recorded during perfusion

During lowered systemic arterial blood pressure to 50 mm Hg, *e g* by an inadvertent bleeding shock the maximal response of the isthmic circular muscles to adrenergic nerve stimulation did not appear to be diminished Neither was any reduced response to nerve stimulation observed when the ampulla was transsected just distal to the IAJ The recorded opening pres

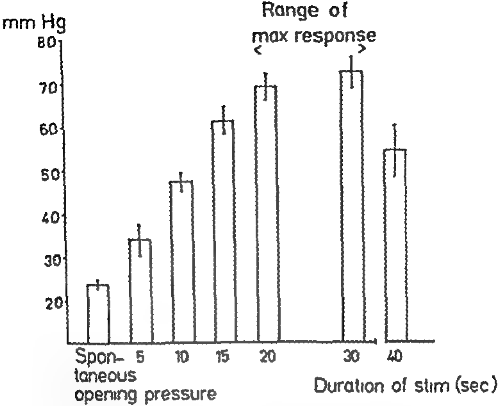

Fig 7 Opening pressures (mean ± S E) at different durations of hypogastric nerve stimulation (20 p/s) during oviduct perfusion in ovarian direction Catheter tip in isthmus 0 5 cm from TUJ Twelve oviducts (5 anestrous and 7 estrous rabbits)

sure usually decreased somewhat during the course of an experiment but intermittent stimulated opening pressures could still be compared over a period of 30—60 min

Relation between stimulation response and frequency

Provided the voltage and the duration of the stimulation were kept constant the pressure response from the isthmus could be varied by the frequency of the electrical stimulation (Fig 8) The influence of the frequency upon the stimulation response was particularly studied in 3 animals in which the frequency range of (1)—3—40 pulses/sec was systematically applied In all of these preparations (1 anestrous and 2 estrous animals) a uniform type of increasing pressure response by increasing stimulation frequency was recorded In one of these animals (anestrous) an increased perfusion pres

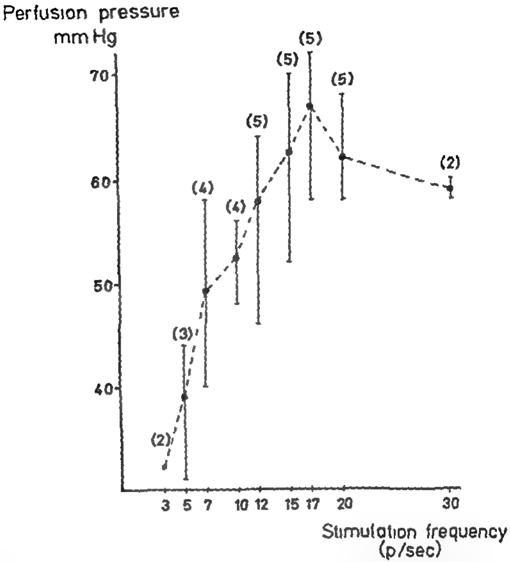

Fig 8 Increase in perfusion pressure (mean and range) at different frequencies of hypogastric nerve stimulation Oviduct perfusion in ovarian direction Catheter tip in isthmus 0 5 cm from TU J Seven animals (3 estrous and 4 anestrous rabbits)

sure was recorded when 1 pulse/sec was used Within the frequency range of 3—7 pulses/sec a certain degree of spontaneous motility remained during the stimulation and occlusive rates of rise of the pressure curve was only irregularly recorded in this range An increasing rate of rise of the perfusion pressure was instead recorded occasionally exhibiting an occlusive rate of rise at the end of the stimulation period The spontaneous motility disappeared gradually as the frequency was increased above 7 pulses/sec and

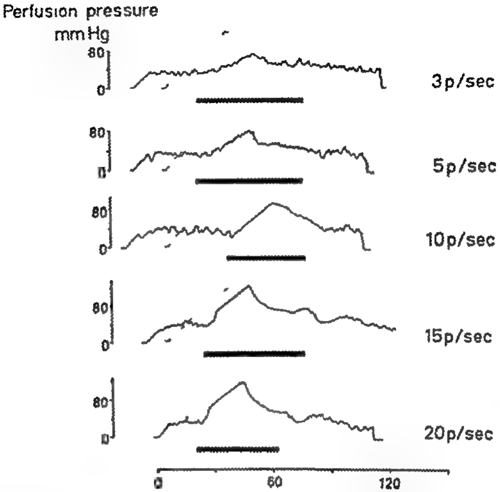

Fig 9 Hypogastric nerve stimulations with increasing frequencies during perfusion (28 µl/min) in ovarian direction in one oviduct Catheter tip in isthmus 0.5 cm from TUJ Stimulation periods indicated by bars Dotted lines occlusive pressure rise Smear atrophy

was completely replaced by an occlusive rate of rise of the pressure curve in the range of 10—15—20 pulses/sec (Fig 9) When frequencies above 20 pulses/sec were used the level of perfusion pressure gradually decreased as the frequency of the stimulation was increased (Fig 8) This was regularly recorded when the stimulation was continued past 25—30 sec (see above) This phenomenon was either faint or not recorded at all in the frequency range of 3—7 pulses/sec Above 7 pulses/sec this escape became more pronounced with increasing frequency No consistent difference

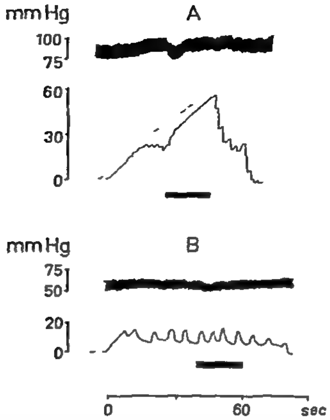

Fig 10 Effect of phentolamine A Hypogastric nerve stimulation (25 p/s) during isthmus perfusion (28 µl/min) in ovarian direction. Catheter tip in isthmus 0 5 cm from TLJ Top tracing systemic arterial blood pressure Immediately after end of perfusion phentolamine (0 5 mg/kg i v) was given.
B The same stimulation test 3 min later is ineffective Dotted lines occlusive pressure rise Smear atrophy

was observed in response to nerve stimulation between anestrous and estrous animals

Pharmacological inhibition of stimulation response

Phentolamine Regitin ©, Ciba Ltd), an *a* blocker known to minimize the peripheral response to adrenergic nerve stimulation on the appropriate receptor was given to 4 animals during the course of the stimulation experiments Two of the animals received 0 5 mg/kg bodyweigth i v and the others 1 0 mg/kg by the same route Phentolamine in these doses caused a decrease of the systemic arterial blood pressure The effect of phentolamine upon the tubal motility is illustrated in Fig 10 Nerve stimulation had

no effect 2 min after the administration of the blocking agent. Some recovery took place after 10 min but complete recovery was not seen until 75 min after the administration of the smaller dose. After 1.0 mg/kg a decrease was observed both in the spontaneous opening pressure, the level of perfusion pressure and the spontaneous motility. Nerve stimulation had no effect on the level of perfusion pressure and only a slight recovery of the stimulation response was observed 90 min after this dose.

Reserpine (Serpasil©, Ciba Ltd) 0.25 mg/kg was given i.v. to 5 rabbits 20 hours before the experiment. This dose had previously been found to reduce the NA content of the rabbit oviduct to amounts not measurable by the method used. This pretreatment did not alter the spontaneous opening pressure during isthmus perfusion which showed a mean value of 32 ± 2.3 mm Hg in 17 estimations. In 3 of the 5 reserpine treated rabbits electrical stimulation of the hypogastric nerves (12 tests) was completely ineffective. In the remaining 2 animals a certain increase of the resistance to flow was recorded during nerve stimulation but no occlusive rate of rise of the pressure curve. After the first 2—3 stimulation periods no further stimulation responses could be obtained. There is good evidence that some adrenergic impulses are still transmitted despite considerable depletion of the NA stores by *e.g.* reserpine (Trendelenburg and Gravenstein 1958, Rosell and Sedvall 1962, Andén 1964). When the remaining NA stores of the adrenergic nerve endings of *e.g.* vasoconstrictor nerves are further reduced by nerve stimulation this results in loss of transmission after a certain number of impulses (cf. Sedvall 1965). The disappearance of the stimulation response from the isthmus after the first 2—3 stimulation periods was probably due to a similar additional stimulation induced depletion of the NA stores remaining after the treatment with reserpine. The spontaneous motility appeared unaffected by the reserpine pretreatment in spite of the reduced or missing nerve stimulation response.

Pentamethonium (Pendiomid©, Ciba Ltd), known as a ganglion blocker with no direct effect on the smooth muscle fiber (Paton and Zaimis 1951), was given i.v. to 2 animals during the course of the experiment. A few minutes after the administration of 4 mg/kg neither the spontaneous resistance to flow nor the spontaneous motility was altered. Electrical stimulation of the hypogastric nerve with a strength which had induced an occlusive rate of rise of the perfusion pressure prior to the administration of the drug was still effective in producing an occlusive increase of the pressure. However, the opening pressure was reduced to about 2/3 of the value recorded before the administration of the ganglionic blocking agent when the same stimulation strength was used (cf. Table III).

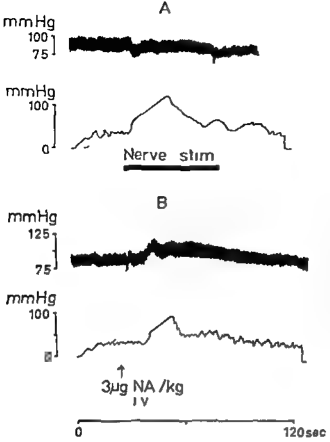

Fig 11 Comparison of hypogastric nerve stimulation and NA administration
A Hypogastric nerve stimulation (25 p/s) during oviduct perfusion (28 μl/min) in ovarian direction Catheter tip in isthmus 0 5 cm from TUJ Top tracing systemic arterial blood pressure
B NA 3μg/kg injected i v 5 min later Dotted lines occlusive pressure rise Smear estrus

Effect of intravenous noradrenaline administration

It may be assumed that the isthmic perfusion pressure response, recorded during stimulation of the adrenergic nerves to the oviduct was mediated by the adrenergic transmittor substance NA In order to test whether the same type of response could be provoked by an increased amount of systemic, circulating NA this amine was injected intravenously to 2 spontaneously anestrous rabbits and 2 rabbits castrated one month before the experiment The perfusion pressure obtained after NA was qualitatively similar to that caused by electrical stimulation of the hypogastric nerves of the same animals (Fig 11) The dose response curves for various NA doses and stimulation frequencies used in one of these experiments are shown in Fig 12 In doses below 0 5 μg/kg, a non occlusive rate of rise was recorded This type of response was recorded after a minimum dose of 0 1 μg/kg No variations of the sensitivity to exogenous NA between the estrous and anestrous ani

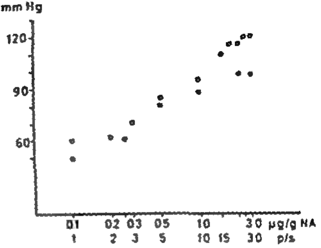

Fig. 12. Dose response curve. Perfusion pressure peaks (ordinate) after increasing doses of i.v. NA (•) and increasing frequencies of hypogastric nerve stimulation (o) in the same preparation. Perfusion (28 µl/min) in ovarian direction. Catheter tip in isthmus 0.5 cm from TUJ. Smear: atrophy.

mals were recorded by the method used. The ampullary perfusion pressure, though rather insensitive to nerve stimulation, did in fact respond to moderate NA doses with short occlusive increases of the perfusion pressure.

Effect of angiotensin induced vasoconstriction

The injection of NA caused an enhanced systemic arterial blood pressure (cf. Fig. 11) and as this effect is due to a peripheral vasoconstriction it might be inferred that the contraction of the circular muscle fibers of the isthmus might have been of hypoxic origin. For that reason the effect of intravenously administered NA was compared to the effect of angiotensin induced vasoconstriction. The effect of angiotensin (Hypertensin ® Ciba Ltd) given by the same route was studied in 6 animals (2 castrates, 2 anestrous and 2 estrous rabbits). Angiotensin did not influence the perfusion pressure of the rabbit isthmus in any of the animals studied in spite of its marked effects on the systemic arterial blood pressure (Fig. 13). For control the reactivity to exogenous NA was tested in the experiments before and after the administration of angiotensin and was found unchanged.

Studies on mated rabbits

Oviducts of 6 rabbits were studied at varying time intervals after mating with one and the same buck known to be fertile. In view of the fact that in the rabbit the eggs are known to pass through the isthmus 75—80 hours after coition (see Introduction) the measurements were done at this time interval. The observations on this material have been compared with those

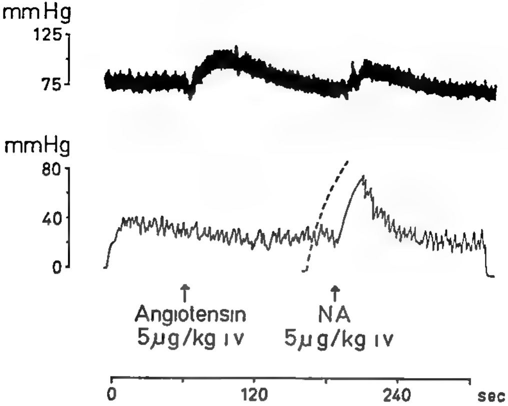

Fig 13 Comparison of perfusion pressure curves after angiotensin and after NA Top tracing systemic arterial blood pressure Oviduct perfusion (28µl/min) in ovarian direction Catheter tip in isthmus 0 5 cm from TUJ Smear estrus

on 4 anestrous non mated controls studied simultaneously The vaginal smears of the mated animals showed influence of estrogen The sensitivity to intravenously administered NA and to electrical stimulation of the hypogastric nerves was studied in both groups Three of the mated rabbits were studied 72—76 hours after coition and the remaining 3 animals 94—98 hours after mating Perfusion from the proximal part of the isthmus in 5 of these animals showed an opening pressure which did not deviate from that of unmated animals However, in the remaining animal, studied 72 hours *post coitum* a spontaneous isthmic opening pressure of 7 3 $\pm$ 0 6 mm Hg was recorded in 7 consecutive estimations In two of the animals (72 and 96 hours *post coitum)* the ampulla was distended by fluid and the abdominal ostia were occluded by the ovaries lying in the infundibuli On repeated tests the opening pressures increased gradually in these animals until the ovaries were removed from the infundibuli After this operation the contents

Table VII Minimum intravenous doses of NA (µg/kg) required to cause detectable and/or occlusive responses in perfusion pressures in anestrous (n = 4) and mated rabbits (n = 6)

Groups of rabbits	Types of responses			
	Detectable response		Occlusive response	
	Mean	Range	Mean	Range
Anestrous (n = 4)	0.5	0.1—1.5	0.73	0.2—1.5
Mated (n = 6)				
72—76 h *post coitum* (n = 3)	4.0	3.0—5.0	4.0	3.0—5.0
94—98 h *post coitum* (n = 3)	5.[illegible]	0.5—10.0	Not obtained	

of the ampullae leaked out through the abdominal ostia and the spontaneous opening pressures subsequently recorded, were of the same magnitude as in non mated rabbits

The response of the isthmus to exogenous NA in the mated animals is compared to that of the anestrous group in Table VII In the mated group the sensitivity tended to be lower than in the control animals and in fact, no occlusive response to the NA doses used was recorded at 94—98 hours after mating

In the mated animals the effect of adrenergic nerve stimulation was about the same at 72—76 and at 94—98 hours after mating The lowest stimulation frequency that induced either detectable or occlusive perfusion pressure responses in the anestrous and mated material is shown in Fig 14 In the mated animals higher stimulation frequency had to be used in order to produce detectable as well as occlusive perfusion pressure responses

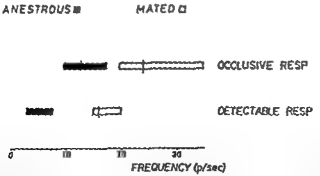

Fig 14 Minimum frequency (mean and range) of hypogastric nerve stimulation causing detectable (≥ 10 mm Hg) and/or occlusive response of perfusion pressure in anestrous rabbits (n = 4) and in animals 72—98 hours after mating (n = 6) Perfusion (28µl/min) in ovarian direction Catheter tip in isthmus 0.5 cm from TUJ

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Perfusion of the oviduct at a constant rate of 16 or 28 μl/min yielded a pressure increase which at first exhibited the same rate of rise as that observed when the catheter tip was mechanically occluded This initial rise ("occlusive pressure rise") was particularly marked when the tip of the catheter was placed in the uterine part of the isthmus The occlusive rate of rise was maintained up to a pressure value of about 30 mm Hg ("opening pressure") at which point the perfusion pressure curve took a horizontal course with spontaneous pressure fluctuations superimposed

The occlusive rate of rise of the perfusion pressure regularly recorded at the start of each perfusion period indicated that the oviduct was occluded during this period The probable cause of this occlusive pressure rise will be discussed later

The isthmic opening pressures recorded during the first few perfusion tests on an oviduct were higher than those obtained at the subsequently repeated perfusions This observation might indicate that at the start of an experiment a "plug" of epithelial cells and/or secretory products was present in the lumen of the oviduct Viscous secretory products would also have to be considered as a possible cause of the relatively high opening pressures recorded throughout an experiment It is known, however that the rate of secretion in the rabbit oviduct is small (Bishop 1956 a, b Clewe and Mastroianni 1960, Hafez 1963) in relations to the perfusion flows used The possibility that secretory products would be an important factor for the resistance to flow and responsible for the high opening pressure may therefore be discarded

The observation that the pressure, required to force the isthmic lumen open was relatively high (31 5 $\pm$ 1 3 mm Hg) both in estrus and anestrus indicates that a certain constrictive tone prevailed in the isthmus An active component in this constrictive tone is suggested by the observation that in one rabbit 72 hours after mating, the opening pressure did not exceed 7 mm Hg during 7 consecutive estimations In previous experiments it had also been observed (Brundin unpublished) that i v injection of prostaglandin

E_1 caused relaxation of the isthmic circular muscles and reduced the opening pressure down to 4—6 mm Hg. This is in accordance with the earlier observation of Horton, Main and Thompson (1963). It is puzzling, however, that prostaglandin E_1 seems to cause contraction of the longitudinal muscles of the isthmus of the human Fallopian tube studied *in vitro* (Sandberg, Ingelman-Sundberg and Rydén 1963).

It could be inferred that the insertion of the catheter in the uterine end of the oviduct and/or the application of the ligature around the oviduct might have caused a constriction of the isthmic circular muscles at the tip of the catheter. This constriction could either be due to direct irritation or mediated by a relative hypoxia. However, in those cases where signs of an impaired circulation through the oviduct appeared, the experiments were discarded. Another possible source of error is temperature, as it is known to influence the degree of contraction in smooth muscles submitted to increased tension. However, between 30 and 40° C this influence does not vary considerably (Csapo 1954). The pH also influences the degree of contraction and reactivity of the smooth muscle as shown in uterine preparations (Halpern, Mayer and Bugnard 1956). If a change in pH did occur in the present studies it would most likely be in the acid direction due to the anesthesia and hence decrease and not increase the muscular reactivity and contractility. A lowered pH could, however, be partly responsible for the gradually decreasing opening pressures normally recorded during prolonged experiments.

From their experiments using the utero-tubal gas insufflation technique Stavorski and Hartman (1958) concluded that the higher the initial flow rate (10—150 cc/min) the higher the resistance to flow offered by the uterine part of the oviduct. However, the two more physiological flow rates of 16 and 28 µl/min used here both yielded the same perfusion pressure. It has previously been reported (Anderson 1927) that the TUJ of the sow offers 4 times higher a resistance to flushing during estrus as compared to that of an animal in anestrus. The same endocrine influence has been observed on the rabbit TUJ by administration of estrogens (Stavorski and Hartman 1958). Feresten and Wimpfheimer (1939) found that the castrated rabbit exhibited a reduced resistance to flow through the TUJ while estrogen pretreatment caused an increased resistance (Wimpfheimer and Feresten 1939). In women a similar reduced resistance has been observed during menopause (Geist, Salmon and Mintz 1938). On the other hand, this endocrine correlation to resistance was not consistent in later studies (Davids and Bender 1940). In the study reported here no results have come forth indicating a difference between the resistance to flow of the isthmus in the estrous and anestrous rabbits. Variations of the resistance to flow of the TUJ

were not recorded in this study because of the positioning of the catheter

It is not possible to draw any definite conclusions from the present series of experiments about the possible adrenergic nervous control of the spontaneous isthmic opening pressure Only in a few cases sectioning of the hypogastric nerves caused an immediate decrease of the perfusion pressure (cf Fig 4) It was also found that neither chronic adrenergic denervation nor reserpine pretreatment caused any significant decreases in perfusion pressure However, it must be emphasized that 1/3 of the normal NA content was still left in the isthmus after denervation Therefore, this operation cannot be considered to have abolished the possible noradrenergic influence on the isthmic muscles Also the reserpine pretreatment probably did not deplete the NA stores extensively enough to exclude some influence of NA upon the muscles of the rabbit isthmus (cf page 35) which is highly sensitive to this amine (Brundin 1964 b) On the other hand, the administration of phentolamine ought to have blocked the α receptors and thus also a possible local NA influence The fact that phentolamine decreased both the isthmic opening pressure and perfusion pressure which furthermore, remained unaffected by nerve stimulation during the influence of this drug indicates that this is due to removal of an adrenergic effect Although an adrenergic control of the sphincteric function of the isthmus thus seems most likely, it may be pointed out that the evidence for this assumption is of an indirect kind, so far However even if the efferent adrenergic impulse traffic to the circular muscles of the isthmus was not present in the majority of the experiments local adrenergic effects might have been sufficient to maintain the muscle in a contracted state In this connection it might be relevant to consider the so called critical closing pressure of small vessels as discussed by Burton (1951)

In many tests the perfusion pressure increased above the value of the opening pressure In these cases spontaneous contractions were superimposed on the perfusion pressure curve (*e g* Fig 3) For obvious reasons no spontaneous pressure fluctuations could be recorded until the perfusion pressure had exceeded the opening pressure The vast discussion in literature on the "peristaltic tubular movements will not be considered in this report (for references see Young 1961) The spontaneous motility of the rabbit oviduct has been held to vary with the endocrine status of the animal (Mikulicz Radecki 1926 Westman 1926 Binder 1939 a b Wimpfheimer and Feresten 1939, Black and Asdell 1958, 1959, Greenwald 1961, 1963, and others) In the study presented here the spontaneous activity varied considerably during the course of a single experiment and no definite conclusions can, therefore be drawn as to whether or not the magnitude of this activity varied with the endocrine status of the animal A probable reason for the more

pronounced variations in spontaneous activity could possibly be the comparatively low rates of flow used in this study as compared to those employed in previous studies. At moderate flow rates the isthmus would not be maximally distended and this would seem to offer more favourable conditions for the recording of the spontaneous activity. The spontaneous circular muscle activity was not affected to any significant degree by hypogastric nerve stimulation at low frequencies (1—7 p/s) but were inhibited at higher frequencies (10—40 p/s).

The statement of Langley and Anderson (1894) that most of the fibers in the hypogastric nerve trunk are non-myelinated later received some support by electrophysiological data. In hypogastric nerves of cats and rabbits slow-conducting fibers of the C group have been demonstrated by Adrian, Bronk and Phillips (1932). They could not detect any B-fibers in the nerves but a small fraction has later been found to show the characteristic properties of B-fibers (Lloyd 1937, Grundfest and Gasser 1938). However, strong evidence has lately been presented that a great number of the hypogastric nerve fibers innervating the male genital organs are preganglionic in many mammalian species (Sjöstrand 1965).

In the papers of Langley and Anderson (1894, 1895, 1896) it was established that the only adrenergic nerves reaching the rabbit oviduct are derived from the hypogastric nerve. Also in the human the hypogastric nerve supplies the oviduct (Mitchell 1938) with adrenergic nerve fibers from the lumbar sympathetic chain but, in addition, it also carries sacral parasympathetic fibers (Learmonth 1931). In the human oviduct the intramural nerve distribution forms a thin homogenous net penetrating the oviduct wall in a uniform manner throughout the entire organ (Damiani and Capodacqua 1961). Neither these authors nor previous workers (e.g. Dahl 1916, Beaufays 1937, Chiara 1959) have demonstrated any ganglion cells in the wall of the human oviduct. Except for those ganglion cells which are known to be situated in the inferior mesenteric ganglia, sending axons also to the oviduct (Langley and Anderson 1894, 1895), no information appears available on the existence or site of any other adrenergic ganglion cells innervating the rabbit oviduct. However, the relatively high amount of NA in the rabbit isthmus after denervation at the level of the inferior mesenteric ganglia (cf. chapt. II) indicates that a considerable number of ganglionic synapses are situated peripherally to the inferior mesenteric ganglia. Since no intramural ganglion cells were observed in the present study these synapses may be situated along the peripheral course of the hypogastric nerves and/or in the immediate vicinity of the effector organ as is the case with the mammalian vas deferens (Sjöstrand 1962).

The functional significance of the innervation to the oviduct does not appear to have been studied more closely. Instead, attention has been paid to variations of the uterine activity that could be elicited by electrical stimulation of the hypogastric nerves (Langley and Anderson 1895, Cushny 1906, Rudolph and Ivy 1930, Rosenblueth 1932, Reynolds 1949, Schofield 1952, Setekleiv 1964 c).

The nature of the transmitter released during stimulation of the hypogastric nerves has been a matter of discussion. The similarity between the uterine responses elicited by hypogastric nerve stimulation and by i.v. administration of A or NA has been taken as an indirect evidence for the response to be of adrenergic origin (Dale 1906, Cushny 1906, Schofield 1952, Cross 1958, Setekleiv 1964 c). The response of the rabbit myometrium *in vivo* elicited by hypogastric nerve stimulation can be blocked by adrenergic blocking agents (Schofield 1952, Setekleiv 1964 c). This also holds for the isthmus of the rabbit oviduct as shown in the present investigation. Recent studies *in vivo* on the effects upon the rabbit myometrium of hypogastric nerve stimulation have not presented any evidence for the presence of cholinergic nerve fibers in the hypogastric nerves to the uterus (Setekleiv 1964 c). The depressed myometrial response to hypogastric nerve stimulation after large doses of atropine as described by Reynolds and Foster (1940) has later been held to depend upon a direct action of the drug upon the myometrium *in vivo* (Schofield 1952). However, Varagic (1956) found that atropine depressed the response to hypogastric nerve stimulation in the isolated rabbit uterus while eserine potentiated the response. In pilot experiments preceding the actual study atropine (20 mg/kg) was found neither to influence the perfusion pressure curve of the rabbit oviduct nor its response to electrical stimulation of the hypogastric nerves. This finding agrees with earlier observations on the rabbit uterus during stimulation of the hypogastric nerves (Cushny 1906).

Though ample information is available on the effect of hypogastric nerve stimulation on uterine motility, very few reports (*e.g.* Kok 1927 a) have been presented about the oviduct in connection to hypogastric nerve stimulation. Even if the oviduct is intimately connected to the myometrial smooth muscle, its response to hypogastric nerve stimulation may be quite different from that of the uterus particularly since the pattern of adrenergic innervation seems to be quite different in the two organs. Both in the rabbit and in the human the myometrium of the uterine horns and the uterine cornua appear to be devoid of adrenergic innervation ending on the smooth muscle fiber (Brundin and Wirsen 1964 a, b) while the smooth muscle fibers of the isthmus are abundantly innervated by adrenergic nerves in both species.

The response elicited by sympathetic nerve stimulation is known to be more widespread in the effector organs than is the response to parasympathetic nerve stimulation. The adrenergic response of the rabbit isthmus also seems to involve the entire isthmus and appears to elicit a mass contraction of the circular muscles. This conclusion is based on direct observation of the organ under a dissection microscope during the functional studies reported above. The histochemical features discussed in chapter II also indirectly support this conclusion.

In the few experiments (e.g. Fig. 6) where a slight pressure increase was recorded from the ampulla during hypogastric nerve stimulation, this increase could be due to contraction of some circular muscle fibers close to the IAJ or, possibly, to a vascular "overflow" of the released transmitter which was found to enhance the ampullary perfusion pressure when administered i.v. The occlusive type of response to adrenergic nerve stimulation consistently recorded from the isthmus when a sufficiently high frequency was used, was never obtained from the ampulla-infundibulum. This may be explained not only by the scarce representation of adrenergic nerves in this part of the oviduct but also by a limited capacity of the relatively thin walled ampullary part to occlude its lumen. On the other hand, the muscular prerequisite of the isthmus (Hermstein 1928, Greenwald 1961) would seem to provide this part of the oviduct with the capacity to occlude completely.

From Fig. 8 it is obvious that the frequency of the stimulation determined the magnitude of the pressure response. In different experiments the optimum response was recorded in the range of 15—25 p/s. Rosenblueth (1932) reported that in the pregnant uterus of the cat the maximum response to hypogastric nerve stimulation was obtained at a stimulation frequency of 20 p/s. The optimum stimulus frequency for the estrogenized rabbit uterus *in vivo* has also been described to occur in the frequency range of 30—50 p/s when the hypogastric nerve is stimulated (Sjöberg 1964 c). In the guinea pig vas deferens the optimum stimulation frequency has been found to be about 30 p/s (Burnstock and Holman 1961).

No information appears to be available from literature about the firing frequency which may occur in the hypogastric nerve fibers to the oviduct during physiological conditions. In studying the response of vascular smooth muscles Folkow (1952) presented indications that the discharge range in the vasomotor nerves only exceptionally exceeded 6—8 p/s. Normal peripheral vascular resistance was maintained at a discharge rate of 1—2 p/s though the use of 15—25 p/s still increased the vascular tone. This additional increase was interpreted as a result of an extremely high output of the transmitter.

for the circular contractile force of the oviduct isthmus The active motility of the uterus during estrus is depressed during progestational conditions as is that of the Fallopian tube (Reynolds 1949, Borell, Nilsson and Westman 1957, Black and Asdell 1958) Thus, it is possible that the tubal muscles causing the constriction of the isthmus, especially observed during the first days after ovulation, may also be influenced by the increasing amount of circulating progesterone, secreted from the growing corpora lutea When the level of circulating progesterone reaches a certain value, the basic constrictive force of the isthmic circular muscles will probably also be decreased, thus allowing the passage of the ova It may be of physiological importance that even if the activity of the adrenergic nerves should be increased during this period, e g as a result of stress induced increase in sympathetic activity, its effect on the isthmus will be less than during other periods of the sexual cycle

From the biochemical, histochemical and functional results presented here, it may be concluded that the rabbit isthmus acts like a sphincter In the estrous and anestrous rabbits studied, the active tone of this sphincter can be modified by the efferent adrenergic impulse traffic in the hypogastric nerve fibers supplying the oviduct It may be pointed out that the distribution of adrenergic nerve terminals in the wall of the human oviduct is similar to that of the rabbit (Brundin and Wirsen 1964 a, b) Also in the human oviduct the circular muscle layer of the isthmus is abundantly penetrated by varicose adrenergic nerve terminals while the adrenergic nerve supply to the ampulla and the infundibulum is more scarce, and mostly located in the perivascular areas This dense adrenergic innervation of the human isthmus, unknown until recently, may be of functional significance with regard to certain gynecological disorders For instance, Meaker (1924) described a group of women, whose oviducts offered high or complete resistance to uterine kymoinsufflation although anatomically normal Since normal kymograms were obtained in these cases after anti spasmodic drug administration, the high resistance was postulated to depend upon tubal spasm Furthermore, Kennedy (1925) described the condition of isthmo-spasm in a certain number of sterile women complaining of dysmenorrhoea The isthmo spasm was held to be due to irregularities of the function of the autonomic nerves to the isthmus and this condition was also thought to predispose for ectopic pregnancies In addition, the succes in treatment of persisten dysmenorrhoea by presacral neurotomy (Cotte 1925) is considered due to the interruption of an increa creased adrenergic influence on the genital smooth muscles (Cotte 1937)

It would thus, seem justified to conclude that the rich adrenergic nervous supply to the human isthmus should be taken into consideration in diagnosis and treatment of certain functional genital disorders

SUMMARY

The distribution and function of the adrenergic nerves in the rabbit oviduct were studied by several experimental procedures.

Catecholamine estimations revealed high amounts of noradrenaline in the isthmus (mean 2.3 μg/g tissue wet wt.) and small amounts in the ampulla-infundibulum (mean 0.3 μg/g tissue wet wt.), indicating that the adrenergic innervation was rich in the isthmus and sparse in the rest of the organ. The content of adrenaline as an indicator of chromaffin cells was negligible throughout the oviduct. Transsection of the hypogastric nerves at the level of the inferior mesenteric ganglia reduced the noradrenaline content of the oviducts by 2/3, indicating that a considerable portion of the adrenergic fibers to the oviduct derives from ganglia located peripherally to the site of denervation. Pretreatment with estrogen and progesterone did not alter the oviduct content of noradrenaline but influenced the weight of the organ.

Histochemical observations showed a rich adrenergic innervation of the isthmic circular muscle layer while that of the ampulla and infundibulum was poor in agreement with the catecholamine estimations.

Functional studies were carried out on anesthetized rabbits. Perfusions of the oviducts with small constant flows of physiological saline permitted estimations of the constrictive activity in different parts of the oviducts. When their opening pressures had been reached the perfusion pressures remained fairly constant.

In non mated estrous and anestrous rabbits a mean isthmic opening pressure of about 30 mm Hg indicated that the isthmus was actively closed. This pressure was not lowered after section of the hypogastric nerves at the level of the inferior mesenteric ganglia, by reserpine pretreatment or after pentamethonium administration. However, blocking of the α receptors by phentolamine decreased the opening pressure of the isthmus.

Electrical stimulation of the hypogastric nerves immediately below the inferior mesenteric ganglia increased the opening pressure of the isthmus and the perfusion pressure of the ampulla-infundibulum. This stimulation effect was weak or moderate at 1—7 p/s and strong at 10—20 p/s. Reserpine pretreatment or phentolamine administration decreased or abolished the stimulation response. Systemic ganglion blocking by pentamethonium caused a reduction of the stimulation response by 1/3, which might be ex-

pected as 1/3 of the noradrenaline content remained in the oviducts after the denervation The various degrees of constriction in the isthmus, obtained at different stimulation frequencies, could be reproduced by different doses of i v noradrenaline injections

In a mated group, studied 72—98 hours *post coitum*, the isthmic contraction responses to nerve stimulation and NA injections were reduced One of six rabbits exhibited a markedly reduced isthmic opening pressure which in the others was similar to that of the non mated rabbits

It is concluded that the isthmus of the rabbit Fallopian tube can be regarded as a sphincter, which probably keeps the isthmic lumen occluded by means of adrenergic mechanisms In non mated rabbits the sphincteric function of the isthmus is very marked and can be further augmented by increased hypogastric nerve activity In mated animals lower opening pressure and smaller effects of nerve stimulation and exogenous noradrenaline can be observed at the time of passage of the fertilized ova through the isthmus

The similarity of the distribution of adrenergic nerves in the rabbit oviduct and in the human Fallopian tube is pointed out and discussed on the basis of certain functional gynecological disorders

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HISTOCHEMICAL AND CYTOPHOTOMETRIC OBSERVATIONS ON ESTERASES IN THE SPINAL GANGLION OF THE RAT

BY
AULIKKI KOKKO

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PREFACE

First of all I should like to acknowledge my great indebtedness to Professor Olavi Eränkö M D Ph D Head of the Department of Anatomy the University of Helsinki under whose guidance the present work was carried out He proposed in 1962 the spinal ganglion as the subject of the present study which is a part of the departmental research project on the nervous tissue directed by him. He has generously advised me in various problems during the whole course of this work. My sincere thanks are offered to him

To Professor Mikko Niemi M D who recently left our Department after being appointed to the Chair of Anatomy in the University of Turku I wish to express my deep gratitude for his interest in my work and for many illustrative valuable discussions

I should like to express my deep gratitude to all my colleagues in the Department of Anatomy for pleasant collaboration and friendly dealings My particular thanks are due to Dr Ulla Soderholm M D who sharing the same laboratory has given me irreplaceable help in many phases of the present work I am also very grateful to Docent Matti Harkonen M D Dr Martti Ikonen M D and Dr Arvi Kahri M L for stimulating discussions which have delayed the publication of the present paper but I hope improved its quality

Dr Archibald Baillie M B Ch B D Sc of the Department of Anatomy the University of Glasgow corrected the English I thank him for pleasant collaboration during his stay as a Visiting Scientist in our Department in Helsinki

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Helsinki October 1965

Aulikki Kokko

CONTENTS

INTRODUCTION

Since Ehrenberg (1833) first described spinal ganglion cells there has been considerable disagreement about their nature function and morphology Although Dogiel (1908) classified these cells into no less than 23 different types most authors would today agree with Scharf (1958) that such classification has little functional meaning

The majority of spinal ganglion cells are pseudounipolar sensory cells Those with a large perikaryon are thought to be concerned with the modalities of touch deep sensation and proprioception Neurones with a small perikaryon on the other hand are thought to be concerned with pain temperature and pressure. These assumptions are based mainly on neurosurgical findings and conclusive evidence is still lacking (Scharf 1958)

The classification of spinal ganglion cells into light and dark ones has long been a source of controversy and it is likely as Fisher and Ranson (1934) point out that most of the »dark cells» of earlier writers are simply cells which have shrunk during fixation and staining However Scharf (1958) in an authoritative review firmly states that a dual morphology exists although he is unable to interpret its meaning

Enzyme histochemistry and electron microscopy have thrown new light on many cytological problems but the nervous system as a whole and especially its sensory part has attracted relatively scant attention Koelle (1962) has made ingenious use of histochemistry in studying cholinergic transmission illustrating the potentialities of a morphological approach in the study of neurone function In addition refined methods are now available for measuring enzyme activity in individual cells and these have also been adapted to study oxidative enzymes in spinal ganglion cells (Hydén 1960)

Important differences in the enzyme activity of adjacent spinal ganglion cells have been observed histochemically but no information is available regarding the histochemical activity of different enzymes in a single ganglion cell Equally the relation between the cell size and enzyme activity is poorly understood and their relation to ganglion cell function is unclear

The present study was undertaken to elucidate the distribution of different carboxylic esterases and acid phosphatase in the spinal ganglion At the same time their possible relationship to the problem of dark and light cells was kept in mind In the course of the work certain hydrolytic enzymes were found to occur in cytoplasmic granules accordingly a fuller study of these granules was undertaken Since an extreme variation in the enzyme activities of individual cells was found a quantitative study was carried out in an attempt to relate hydrolytic enzymes with one another and with the cell size

MATERIAL

About 400 male and female adult rats of Sprague Dawley strain were used The animals were killed by decapitation while under ether anaesthesia The spinal ganglia were removed by a dorsal approach through the vertebral arches and after dissecting out the spinal cord the intervertebral foramina were explored The ganglia from all the segments were studied Since no es ential regional differences were found the cervical ganglia were preferred on account of their larger size

HANDLING OF THE GANGLIA

After removal the ganglia were either fixed in 4 % formaldehyde containing 1.2 % calcium chloride for three hours at room temperature or frozen fresh in liquid air and mounted in a drop of 10 % gelatin on a steel tissue holder The fixed ganglia were cut on a freezing microtome in 6—10 μ and handled thereafter as free floating sections Fresh frozen sections were cut in a cryostat at 6—10 μ, mounted on clear coverslips and allowed to dry at room temperature

For electrophores s a large number of spinal ganglia was collected homogenized in an all gla s homogenizer containing 4 mg fresh tissue /10 μl distilled water and the homogenate frozen and thawed six times prior to centrifugation at 25 000 × g at 4 C for 30 mins Twenty μl of the clear supernatant was used for each run of electrophoresis

I CARBOXYLIC ESTERASES

LITERATURE REVIEW

ACETYLCHOLINESTERASE

Acetylcholinesterase in the Spinal Ganglia

Acetylcholinesterase (AChE) is the most widely studied and best understood group of carboxylic esterases and its place in nervous transmission has been reviewed recently by Koelle (1962) In his opinion acetylcholine (ACh) is the »original» transmitter both in cholinergic and non-cholinergic neurones regardless of the main transmitter substance This view is supported by Nachmansohn s findings (1959) that AChE exists in all nervous tissues

The spinal ganglion is thought to have a weak or moderate AChE activity Koelle (1951 1955b) observed extremely low activity in cat and rat dorsal root ganglia and noted that the nerve fibres in the ganglia were very faintly stained In his studies on the central nervous system (1954 1955a) he generally emphasized the low activity or absence of AChE in primary sensory neurones Koelle therefore postulated that sensory neurones are non-cholinergic a substance other than ACh being mainly responsible for nervous transmission

Gerebtzoff (1959) also reported a low overall generalized AChE activity in spinal ganglia and drew attention to considerable variations in AChE activity of individual ganglion cells in the spinal ganglion of the guinea pig and rabbit In the dog on the other hand Gerebtzoff found the AChE activity restricted to the area immediately inside the pericellular capsule.

AChE in developing spinal ganglia of the chicken embryos was studied by Strumia and Baima Bollone (1964) who were able to demonstrate the enzyme in 4-day old embryos Okinaka *et al* (1963) recorded little activity in the dorsal root ganglia of man the dog and the horse

On the other hand, all the spinal ganglion cells and fibres of the rat were found to exhibit positive AChE reaction by Coupland and Holmes (1957) Cauna and Naik (1963) likewise reported that the cytoplasm of the sensory nerve cells contains AChE in all the species which they studied (cat, guinea pig rat mole and man) although the intensity varied from one cell to another In a series of papers Tewari and Bourne (1962a and c) reported considerable variations in the intensity of the AChE activity in individual ganglion cells in the rat They recognized small cells which always show a positive cytoplasmic reaction which tends to be perinuclear Some large cells seemed to be entirely negative others

showed great variations both in the intensity and in the distribution of the reaction. In some cells the reaction was finely granular, in others coarse. In cells with the coarse granular reaction the activity was concentrated in one side of the cell in a broad area near the cell membrane. Not only the cytoplasm was positive in these cells but a positive reaction was observed at the rim of the nucleolus. This is the only recent report of nuclear AChE. A nuclear reaction was often reported in early studies made with inadequate techniques (see the review by Esila 1963).

Giacobini (1956, 1959a and b, 1960) studied isolated spinal ganglion cells of the rat with histochemical and microgasometric methods. He observed that while 80—90 % of the cell population exhibit only slight or no AChE activity, about 10—15 % exhibit an intense activity. No morphological differences were found between the two groups of cells and they were anatomically mixed in the ganglion. In the spinal ganglion axon Giacobini (1959a and b) found activity 10—100 times greater than in the cell body, which is in marked contrast with the findings in motor cells.

Intracellular Localization

Of the functional units participating in cholinergic transmission, AChE is the only enzyme which can be accurately localized histochemically. Fukuda (1959) and Fukuda and Koelle (1959) studied the distribution of AChE in cat's ciliary ganglion with histochemical and histological methods and came to the conclusion that the distribution of AChE corresponds with that of the Nissl substance. On this basis they suggested that AChE is synthesised in the endoplasmic reticulum and then transported via its cisternae to the surface of the cell and to its processes. These observations are supported by the biochemical studies of Toschi (1959) and Hanzon and Toschi (1959) who found that the highest AChE activity in subcellular fractions of the rat brain obtained by differential centrifugation is associated with the microsomal fraction. Further centrifugation of this fraction resulted in a pure membrane fraction and a pure granule fraction (as judged with the aid of the electron microscope). The granule fraction contained a high percentage of ribonucleic acid (RNA) and the membrane fraction most of the cholinesterase (ChE) activity of the microsomal fraction. Aldridge and Johnson (1959) also found the highest AChE activity in the microsomal fraction of centrifuged brain homogenate. However, they observed that the mitochondrial fraction as well contains remarkably high AChE activity. They attributed this to microsomal contamination. De Robertis and Whittaker have separately studied the subcellular distribution of AChE, ACh and cholinacetylase (ChAc) both biochemically and with the aid of the electron microscope. According to both, the microsomal fraction of both the rat and the guinea pig brain contains high AChE activity, but the high AChE activity in mitochondrial fraction is not due to microsomal contamination; instead subfractionation shows the existence of synaptosomes in which ACh is localized preferentially in the synaptic vesicles while AChE occurs in the membranous part of the nerve ending. De Robertis' group considers that ChAc is also associated with the synaptic vesicles, probably with their outer surface, but Whittaker's group has suggested

a free cytoplasmic localization of ChAc (Whittaker 1959 Gray and Whittaker, 1960 Whittaker *et al* 1964 De Robertis *et al*, 1961, 1962a and b 1963 Rodriguez De Lores Arnaiz 1964) The localization of AChE in the endoplasmic reticulum and synapses more particularly in the pre and postsynaptic membranes gains further support from the electron microscope histochemistry of Torack and Barrnett (1962) and Lewis and Shute (1964)

Giacobini (1957, 1959a 1960) has studied AChE in small fragments of nerve cells with histochemical and microgasometric methods In the large anterior horn cell of the spinal cord of the rat the perikaryon and the dendrites exhibit more activity than the axon The nucleus exhibits little activity and the nucleolus none

NON-SPECIFIC CHOLINESTERASE

In most studies concerning spinal ganglia non specific cholinesterase (ns ChE) has exclusively been localized in capsular glial cells and nerve fibres, presumably the Schwann cells (Koelle 1951 1955b Coupland and Holmes 1957) the animals studied were man the cat the dog the rabbit the rat and the Rhesus monkey Giacobini s findings (1959a and b) also favour a glial localization but he also noted some ns ChE activity in a few neurones of the spinal ganglion

Cauna and Naik (1963) were not able to find any ns ChE activity in the spinal ganglia of the rat although with the same technique the tissue elements surrounding the nerve cells and nerve bundles in the cat spinal ganglia exhibited intense activity Similarly Tewari and Bourne (1962a) reported that the spinal ganglia of the rat are totally devoid of ns ChE activity

NON SPECIFIC ESTERASES

In the earlier studies concerning the non specific esterases (ns E) the division between esterases inhibited by diethyl p-nitrophenyl phosphate (E 600) *i e* E 600 sensitive non specific esterases (E s ns E) and E 600 resistant non specific esterases (E r ns E) has not always been taken into consideration On the other hand in some publications there has appeared a more detailed classification based on the use of various substrates inhibitors and activators

Non specific Esterases of Spinal Ganglia

Only a few reports have been published on the ns E activity of the dorsal root ganglia Tewari and Bourne (1962a) studied the distribution of ns E in frozen sections of rat spinal ganglia with substituted naphthols as substrates They found large variations in respect of the activity between individual nerve cells some being negative others intensely positive The reaction product was intracellular and finely granular Some cells showed broad perinuclear activity

while in others there was a diffuse cytoplasmic reaction superimposed on the granular reaction

Thomas (1963) considered 4 chloro 5 bromoindoxyl acetate as the substrate of choice in fresh sections of human spinal ganglia and described individual variations in the reaction intensity in different ganglion cells The reaction was diffuse but more intensely stained granules occurred which he assumed to be mitochondria There was also an intense reaction associated with lipofuscin pigment The satellite cells were also reactive though the reaction was less intense than that observed in the ganglion cells Some fibres in the nerve bundles exhibited a positive reaction assumed by Thomas to be intra axonal

Intracellular Localization

Using the substrate phenyl butyrate following differential centrifugation the ns E activity of rat brain was estimated by Aldridge and Johnson (1959) The observed activity was not inhibited by eserine and was therefore probably due to ns E The distribution of ns E in subcellular fractions somewhat resembled that of ChE Both mitochondrial and microsomal fractions had high ns E activity but the most intense activity was seen in the microsomal fraction With o nitrophenyl acetate on the other hand as substrate in the rat cerebral cortex the highest specific activity resided in the microsomal fraction (Sellinger and De Balbian Verster 1962) and their observations agree with those of Aldridge and Johnson They did not however exclude the possibility that AChE hydrolysed this substrate

Torack and Barrnett (1962) studied with the aid of the electron microscope the intracellular localization of esterases in the rat brain stem using thiolacetic acid and eserine Ns E activity was found in the endoplasmic reticulum and Golgi complex of neurones although these structures showed less intense activity than certain dense cytoplasmic bodies presumed to be lysosomes This was also true of glial cells It is interesting to note in this connexion that organophosphorous resistant ns E has been located histochemically in lysosomes in other non nervous tissues (Holt 1963)

ELECTROPHORESIS OF ESTERASES

Hunter and Markert (1957) first separated esterases using starch gel electrophoresis and drew attention to the species specificity and organ specificity of the esterases (see Markert and Hunter, 1959 Lawrence *et al* 1960 Hunter *et al* 1961 Paul and Fottrell 1961 and Hess *et al* 1963)

In 1958 Allen *et al* noted differences between the enzyme spectra in adrenal zymograms and the enzyme distribution in the medulla While eserine almost entirely abolished the reaction obtained in sections with α naphthyl acetate it had little effect on the zymogram Using sections instead of tissue homogenate for electrophoresis it has been established (Eranko *et al* 1962b) that most of the esterase activity demonstrable in an untreated section remains in the section

after electrophoresis although enough soluble esterase has been removed to produce zymograms identical with those obtained with tissue homogenate From these observations it was concluded that the esterases responsible for the activity in the zymograms are readily soluble i e lyo-esterases while the reaction obtained in fresh sections is in part due to desmo-esterases which are firmly attached to tissues In a later study in which zymograms of readily soluble esterases were compared with firmly bound esterases which had been solubilized definite differences in the enzyme spectra were observed (Eranko *et al* 1964)

Formalin immobilizes lyo-esterase but at the same time destroys esterase activity (Hannibal and Nachlas 1959 Eränkö *et al* 1964 Harkonen 1964 Soderholm, 1965) Electrophoresis of formalin fixed material yields fewer fuzzy bands in different positions compared with zymograms of fresh sections (Burstone 1958 Eränkö *et al* 1962b)

Differences in zymograms obtained with different substrates in some non nervous tissues were observed by Hunter and Burstone (1960) and Allen and Hunter (1960) Differing reaction rates rather than real substrate specificity were considered responsible however Pronounced substrate specificity occurs especially in nervous tissues (Eranko *et al* 1962a and b Esila 1963 Söderholm 1965) Substrate preference though not true specificity has also been demonstrated in nervous tissue by Harkonen (1964)

Characterization of the human brain esterases has been done by Barron *et al* (1961 1963) and Bernsohn *et al* (1962)

Several types of enzyme activity have been demonstrated in one zymogram band (see Barron *et al* 1963 Hunter *et al* 1964 Harkonen, 1964 and Soderholm 1965)

METHODS

HISTOCHEMICAL STAINING METHODS

Gomori s (1952) modification of Koelle s (1951) method for cholinesterases was used. The incubation time for sections was 3—4 hours for zymograms overnight at 37 C. To get reasonable results in the latter case thorough rinsing in several changes of Na_2SO_4 solution for two hours was found necessary

α Naphthyl acetate was used according to Pearse (1960) with a saturated solution of Fast Blue RR as coupling agent. Fresh sections were incubated for 3 mins fixed sections for 5 mins at room temperature. Starch slabs were incubated in a coupler concentration of 1 mg/ml for 30—45 mins.

With α naphthyl butyrate the procedure was similar but the incubation time was prolonged to 15 mins for fresh sections and 10 mins for fixed ones and to one hour for slabs at room temperature Fast Blue RR served as coupling agent as described above

Naphthol AS D acetate was used as described by Gossner (1958) with Fast Blue RR as coupling agent. Sections were incubated for one hour at room temperature slabs for 3—4 hours at 37 C.

4-Chloro-5-bromoindoxyl acetate work was based Holt s (1958) method. For staining of starch slabs the concentration of ferri ferrocyanide was reduced to 0 5 mM (Shnitka and Seligman, 1961 Barron *et al* 1963) Sections were incubated at 37 C for 30—60 mins and slabs for 3—4 hours

The methyl green-pyronin staining was carried out as given by Pearse (1960)

ELECTROPHORESIS

Electrophoresis was performed by the method of Markert and Hunter (1959) as modified by Eranko *et al* (1962b) Usually two samples were placed in the same starch slab at 8 cm interval

INHIBITORS FOR THE CLASSIFICATION OF ESTERASES

As in previous studies from the present laboratory (Eranko *et al* 1964 Harkonen 1964 Soderholm 1965) the carboxylic esterases were classified into four groups 1) acetylcholinesterase (AChE) 2) non specific cholinesterase (ns ChE) 3) E 600 sensitive non specific esterase (E s ns E) and 4) E 600 resistant non specific esterase (E r ns E)

To inhibit AChE selectively 1 5 bis-(4 allyl dimethylammoniumphenyl)pentan 3 one-diiodide (284 C 51) was used (Holmstedt 1957) Since organ and species variations in the response of esterases may occur to various inhibitors and substrates (Aldridge 1953a) the optimum concentration of all the inhibitors were checked in preliminary studies 284 C 51 was surveyed in the ranges 10^{-4}M—10^{-7}M and a concentration of 10^{-5}M was routinely used

To inhibit ns ChE selectively tetra isopropylpyrophosphoramide (*iso*-OMPA) was used (Aldridge 1953a Austin and Berry 1953 Bayliss and Todrick 1956) The concentration range surveyed was 10^{-3}M—10^{-8}M When thiocholine was used as substrate the concentration was limited to 10^{-6}M since higher concentrations inhibit AChE to some extent Concentrations of 10^{-7}M or more were high enough to inhibit ns ChE

Eserine was used *to inhibit ChEs selectively* (Richter and Croft 1942 Aldridge 1953b Allen *et al* 1958) Concentration ranges surveyed were 10^{-3}M to 10^{-7}M eserine a concentration of 10^{-5}M was found most suitable

To distinguish between E s ns E and E r ns E diethyl p nitrophenyl phosphate (E 600) was used (Aldridge 1953b Pepler and Pearse 1957 Bergman *et al* 1957 Eranko *et al* 1962b c 1964) The concentrations examined were from 10^{-3}M to 10^{-7}M and the optimum concentration routinely used was 10^{-4}M

Sections and slabs were always preincubated for 20—30 mins in buffer containing the same concentration of inhibitor as the substrate mixture

RESULTS

SECTIONS

For demonstration of ChEs the thiocholine methods proved best. α-Naphthyl acetate, butyrate and indoxyl acetate were also hydrolysed by ChEs but the reaction obtained with these substrates could not be satisfactorily differentiated from that due to ns E.

In demonstrating E-s ns E there was no obvious difference observed between α-naphthyl acetate, α-naphthyl butyrate and 4-chloro-5-bromoindoxyl acetate except that the latter two were more slowly hydrolysed and thus required longer incubation to give the same results as α-naphthyl acetate. Therefore the last mentioned substrate was preferred. Naphthol AS-D acetate gave weak and and diffuse staining and was accordingly discarded.

To demonstrate E-r ns E all the substrates except the thiocholines gave a similar picture. However, again α-naphthyl acetate was preferred because of its shorter incubation time. None of the inhibitors seemed to have any effect on the reaction obtained with naphthol AS-D acetate in fixed sections and it was concluded that this substrate was almost selectively split by E-r ns E. However, the reaction obtained with this substrate was poorly localized and thus it could not be used in preference to the others.

The best way to demonstrate the four groups of carboxylic esterases in tissue sections is summarized in the following scheme.

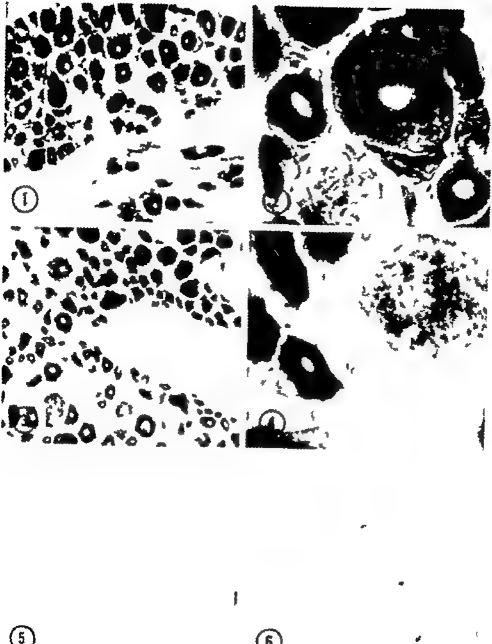
Enzyme	Type of section	Substrate	Inhibitor
AChE	Fresh or fixed	Acetylthiocholine iodide	*iso*-OMPA 10^{-7}M
Ns ChE	Fresh or fixed	Butyrylthiocholine iodide	284 C 51 10^{-5}M
E-s ns E	Fresh	α-Naphthyl acetate	eserine 10^{-5}M
E-r ns E	Fixed	α-Naphthyl acetate	E 600 10^{-6}M

The results obtained with different substrate-inhibitor combinations are summarized in Table 1 for fresh sections and in Table 2 for fixed sections. Negative reaction is labelled —, just visible ±, weak +, moderate ++ and strong +++. Although the effect of every inhibitor on the substrate used has been listed in these tables, they cannot give a complete picture because of the presence of differences in the intensity between the individual cells in a non-inhibited section.

The effect of eserine and E 600 on the reaction obtained with α-naphthyl acetate and Fast Blue RR both in fresh and in formalin-fixed sections is shown in Figs. 1—12.

TABLE 1 *Esterase reactions obtained with different substrate inhibitor combinations in fresh sections*

Substrate and Inhibitor	Ganglion Cells		Satellite Cells	
	Background	Granules	Background	Granules
Acetylthiocholine	— to +++	—	+ to +++	—
iso-OMPA 10^{-7}M	— to +++	—	—	—
284 C 51 10^{-5}M	— to ±	—	+ to +++	—
eserine 10^{-5}M	—	—	—	—
E 600 10^{-5}M	—	—	—	—
Butyrylthiocholine	— to ±	—	+ to +++	—
iso-OMPA 10^{-7}M	—	—	—	—
284 C 51 10^{-5}M	— to ±	—	+ to +++	—
eserine 10^{-5}M	—	—	—	—
E 600 10^{-5}M	—	—	—	—
α Naphthyl acetate	+ to +++	+++	+ to +++	—
iso-OMPA 10^{-5}M	+ to +++	+++	— to ++	—
284 C 51 10^{-5}M	+ to +++	+++	+ to +++	—
eserine 10^{-5}M	+ to +++	+++	— to ++	—
E 600 10^{-5}M	—	—	—	—
α Naphthyl butyrate	+ to +++	+++	+ to ++	—
iso-OMPA 10^{-4}M	+ to +++	+++	— to ++	—
284 C 51 10^{-5}M	+ to +++	+++	+ to ++	—
eserine 10^{-5}M	+ to +++	+++	— to ++	—
E 600 10^{-5}M	—	—	—	—
4-Chloro-5 bromo-indoxyl acetate	+ to +++	+++	+ to +++	—
iso-OMPA 10^{-} M	+ to +++	+++	— to ++	—
284 C 51 10^{-5}M	+ to +++	+++	+ to +++	—
eserine 10^{-5}M	+ to +++	+++	— to ++	—
E 600 10^{-} M	—	—	—	—
Naphthol AS D acetate	Reaction was so weak and diffuse that accurate localization was impossible			



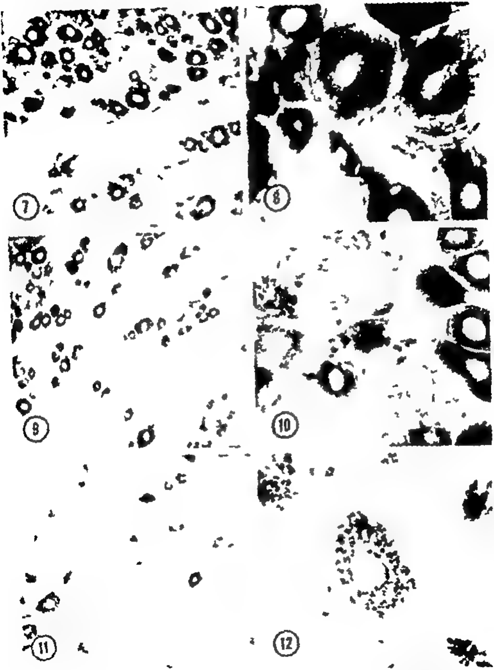
Figs 1-6 Esterase reactions obtained with α naphthyl acetate and Fast Blue RR in fresh sections

Fig 1 No inhibitor × 120
Fig 3 Eserine 10^{-4} M × 120
Fig 5 E 600 10^{-6} M × 120

Fig 2 No inhibitor × 480
Fig 4 Eserine 10^{-4} M × 480
Fig 6 E 600 10^{-6} M × 480

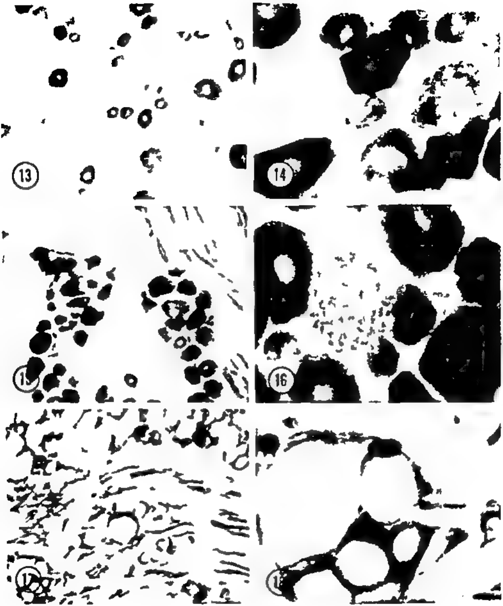
TABLE 2. *Esterase reactions obtained with different substrate inhibitor combinations in fixed sections*

Substrate and Inhibitor	Ganglion Cells		Satellite Cells	
	Background	Granules	Background	Granules
Acetylthiocholine	— to +++	—	+ to +++	—
iso-OMPA 10^{-7}M	— to +++	—	—	—
284 C 51 10^{-5}M	± only few	—	+ to +++	—
eserine 10^{-5}M	—	—	—	—
E 600 10^{-5}M	—	—	—	—
Butyrylthiocholine	± only few	—	+ to +++	—
iso-OMPA 10^{-7}M	—	—	—	—
284 C 51 10^{-5}M	± only few	—	+ to +++	—
eserine 10^{-5}M	—	—	—	—
E 600 10^{-5}M	—	—	—	—
α Naphthyl acetate	+ to +++	+++	+ to +++	+++
iso OMPA 10^{-5}M	+ to +++	+++	+ to ++	+++
284 C 51 10^{-5}M	+ to +++	+++	+ to +++	+++
eserine 10^{-5}M	+ to +++	+++	+ to ++	+++
E 600 10^{-5}M	— to +	+++	— to +	+++
α Naphthyl butyrate	+ to +++	+++	+ to ++	+++
iso-OMPA 10^{-5}M	+ to +++	+++	+ to ++	+++
284 C 51 10^{-5}M	+ to +++	+++	+ to ++	+++
eserine 10^{-5}M	+ to +++	+++	+ to ++	+++
E 600 10^{-5}M	— to +	+++	— to +	+++
4-Chloro-5 bromo-indoxyl acetate	— to +++	+++	+ to +++	+++
iso-OMPA 10^{-5}M	— to +++	+++	+ to ++	+++
284 C 51 10^{-5}M	— to +++	+++	+ to +++	+++
eserine 10^{-5}M	— to +++	+++	+ to ++	+++
E 600 10^{-5}M	— to +	+++	— to +	+++
Naphthol AS D acetate	— to ++	+++	— to +	+++
iso-OMPA 10^{-5}M	— to ++	+++	— to +	+++
284 C 51 10^{-5}M	— to ++	+++	— to +	+++
eserine 10^{-5}M	— to ++	+++	— to +	+++
E 600 10^{-5}M	— to ++	+++	— to +	+++



Figs 12 Esterase reactions obtained with α-naphthyl acetate and Fast Blue RR in fixed sections

Fig 7 No inhibitor × 120
Fig 8 No inhibitor × 480
Fig 9 Eserine 10^{-4}M × 120
Fig 10 Eserine 10^{-4}M × 480
Fig 11 E 600 10^{-4}M × 120
Fig 12 E 600 10^{-4}M × 480



Figs 13–18 Cholinesterases

Fig 13 AChE fresh section. Acetylthiocholine *iso*-OMPA 10^{-7}M × 120

Fig 14 As *Fig 13* × 480

Fig 15 AChE fixed section. Acetylthiocholine *iso*-OMPA 10^{-7}M × 120

Fig 16 As *Fig 15* × 480

Fig 17 Ns ChE fixed section Butyrylthiocholine 284 C 51 10^{-6}M × 120

Fig 18 As *Fig 17* × 480

Acetylcholinesterase (AChE)

In fresh sections the reaction was mainly limited to the ganglion cell cytoplasm (Figs 13 and 14) The cytoplasmic reaction was diffuse or very finely granular and the intensity of the reaction fairly even in each cell The nucleus and nucleolus were always negative as were the satellite cells which appeared as staining defects in the reactive cytoplasm of the ganglion cells Although the activity in a given ganglion cell was uniform there was great variation in the reaction intensity between different ganglion cells AChE-negative cells were picked out by counter staining such as with methyl green pyronin

Few nerve fibres were positive mostly in the centre of the ganglion or in the fibre trunks leading to or from the ganglion never within the nerve cell aggregations

Sections from formalin fixed ganglia resembled fresh sections excepting that the reaction product in cytoplasm seemed to be more granular in character occurring sometimes in clusters (Figs 15 and 16) Variation in the reaction intensity of different cells was more pronounced in fixed than in fresh sections

The activity seemed in general to be higher in the smaller than in the larger cells both in fresh and in fixed sections The amount of weakly or moderately reacting cells exceeded that of the strongly reactive cells Cells of different reactivity were randomly intermingled in the ganglion

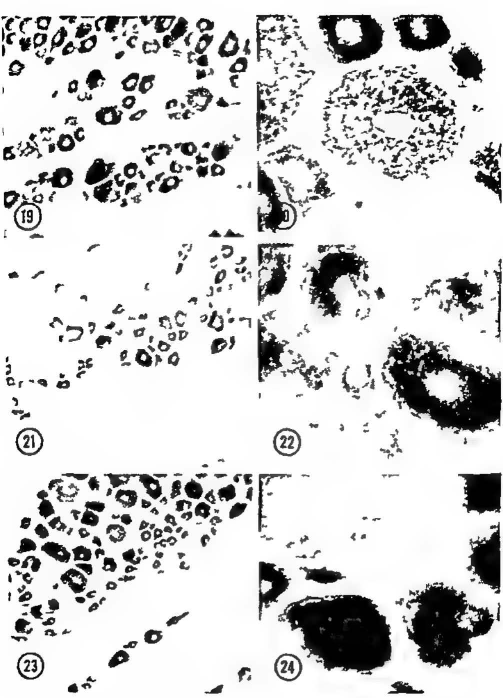
284 C 51 in a concentration of 10^{-5}M inhibited totally the AChE activity from both fresh and fixed sections as did 10^{-5}M eserine and E 600

Non specific Cholinesterase (ns ChE)

Fresh and formalin fixed sections were similar very few ganglion cells showed any activity and the reaction was weak the cells being stained pale yellow (Figs 17 and 18) An intense ns ChE activity was observed in the structures between the ganglion cells These ns ChE positive tissues included capillaries nerve fibres satellite cells and the Schwann cells Most but not all satellite cells showed intense activity although the nuclei were negative The glomerulus was particularly rich in ns ChE activity probably because of the intense reaction in the satellite cells around the tortuous axon leaving the cell body Nerve fibres surrounding the ganglion cells and those in the central nerve trunk of the ganglion showed intense activity, probably due to activity in the satellite cells rather than to axonal activity Part of the capsule enclosing the whole ganglion showed some activity *Iso* OMPA 10^{-7}M eserine 10^{-5}M and E 600 10^{-5}M totally abolished the activity

E 600-sensitive Non specific Esterase (E s ns E)

This type of esterases was best preserved in fresh sections and such sections stained in the presence of eserine 10^{-5}M (Figs 19 20 21 22 23 and 24) E s ns E was localized in the cytoplasm of the ganglion cells their nuclei and nucleoli were not reactive E-s ns E activity was observed in all ganglion cells but distinct variations were observed in the reaction intensity between individual cells



Figs 19–24 Non-specific esterases in fresh sections

Fig 19 α-Naphthyl acetate eserine 10^{-4}M ×120

Fig 20 As *Fig* 19 ×480

Fig 21 α-Naphthyl butyrate eserine 10^{-4}M ×120

Fig 22 As *Fig* 21 ×480

Fig 23 4-Chloro-5-bromoindoxyl acetate eserine 10^{-4}M ×120

Fig 24 As *Fig* 23 ×480

(Figs 19 21 and 23) Most showed even diffuse background staining at low magnifications At a higher magnification however this diffuse reaction exhibited a Nissl like pattern seen in methyl green pyronin stained sections More intensely stained areas alternated with less intensely stained ones (see Figs 20 and 43) In addition to the positive structures just described all cells contained small granules which exhibited a stronger E-s ns E reaction than the background These granules were sometimes evenly distributed over the whole cell (Figs 20, 22 and 24) sometimes aggregated at the cell boundary (Fig 24)

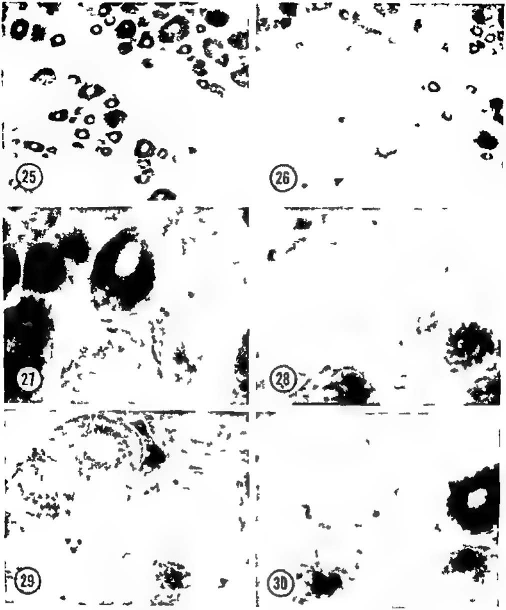
The cytoplasm of the satellite cells was positive but usually less intensely reactive than that in the related ganglion cell The nerve fibres did not usually show any activity Occasionally a weak reaction was seen in the glomerular regions, occasionally in the interstitium between the ganglion cell bodies This activity was apparently due to the satellite or Schwann cell activity In the capsule surrounding the whole ganglion some E s ns E activity was also visible

A fixed section incubated in the presence of eserine 10^{-5}M contained both E s ns E and E r ns E the latter predominating However when fixed sections incubated in the presence of 10^{-5}M eserine (Figs 25 27 and 29) were compared with those incubated in the presence of E 600 10^{-5}M (Figs 26 28 and 30) the latter were found less intensely stained indicating the presence of E s ns E in the former The distribution of E s ns E in fixed sections must therefore be assessed by substracting from the activity of a section incubated in the presence of eserine the activity seen in a section incubated in the presence of E 600 (Figs 25—26 Figs 27—28 and Figs 29—30) This kind of substraction indicated that the reaction product attributable to E s ns E was diffuse or very finely granular and was evenly distributed in the cytoplasm of the ganglion cells The nuclei and nucleoli were negative Also the satellite cell cytoplasm showed activity as did some parts of the capsule encircling the whole ganglion The nerve fibres were negative

E 600 resistant Non specific Esterase (E r ns E)

There was no activity of this kind in a fresh section with any of the substrates used (Figs 31 and 32) Not all the ganglion cells showed E r ns E activity in fixed sections a few cells seemed to be entirely non reactive (Figs 33 35 36 and 38) In the positive nerve cells the reaction product was mostly situated as a broad ring around the nucleus Usually this was composed of fine cytoplasmic granules but occasionally large granules occurred (Figs 33 35 36 37 39 and 40) Some of the cells showed positive coarse granules dispersed throughout the cytoplasm (Figs 33 and 34) In other nerve cells these cytoplasmic granules were gathered to one side of the cell (Figs 33 34 and 40) The nuclei and nucleoli were always devoid of activity

Moreover in most of the satellite cells there were very active cytoplasmic granules often arranged round the nucleus forming a kind of »cap» (Figs 33 34 36 37 and 38) Again in the interstitium outside the satellite cell mantle in connexion with the nerve fibres there was some E-r ns E activity probably in the cells of Schwann or in the capillary pericytes The connective tissue capsule contained sometimes E r ns E activity too



Figs 25 30 Non specific esterases in fixed sections

Fig 25 α Naphthyl acetate
eserine 10^{-6}M × 120

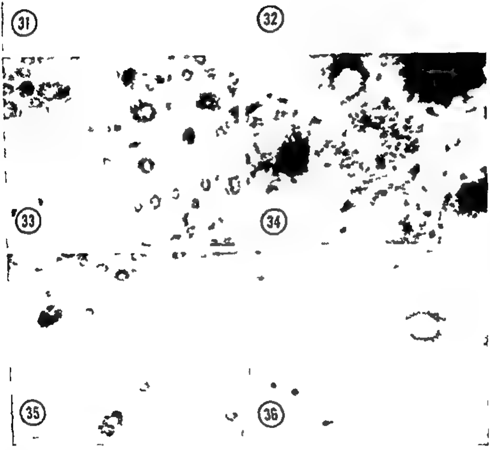
Fig 27 α Naphthyl acetate
eserine 10^{-6}M × 480

Fig 29 α Naphthyl butyrate
eserine 10^{-5}M × 480

Fig 26 α Naphthyl acetate
E 600 10^{-6}M × 120

Fig 28 α Naphthyl acetate
E 600 10^{-6}M × 480

Fig 30 α Naphthyl butyrate
E 600 10^{-6}M × 480



Figs 31 36 Non specific esterases

Fig 31 α Naphthyl acetate E 600 10^{-5}M
Reaction absent in a fresh section. ×120

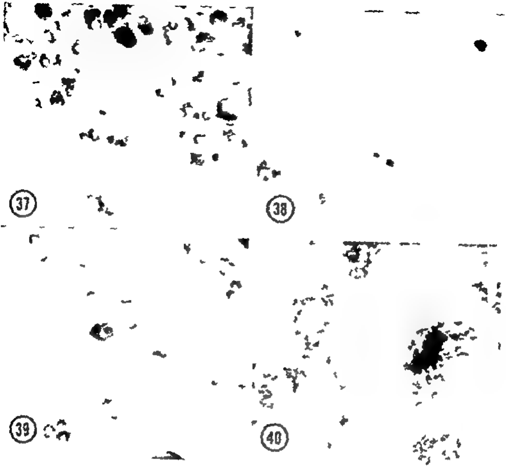
Fig 32 As *Fig 31* × 480

Fig 33 α Naphthyl acetate E 600 10^{-5}M
Fixed section ×120

Fig 34 As *Fig 33* ×480

Fig 35 α Naphthyl butyrate E 600 10^{-5}M
Fixed section ×120

Fig 36 As *Fig 35* ×480



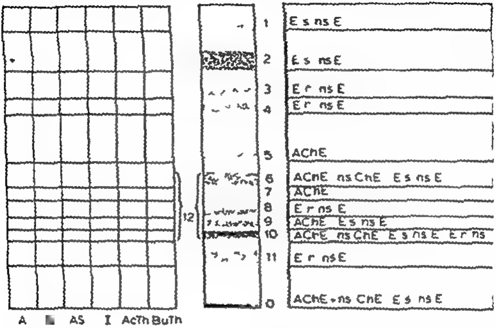
Figs 37–40 Non-specific esterases in fixed sections

Fig 37 4-Chloro-5-bromoindoxyl acetate E 600 10^{-4}M ×120
Fig 38 4-Chloro-5-bromoindoxyl acetate E 600 10^{-4}M ×480
Fig 39 Naphthol AS D acetate E 600 10^{-4}M ×120
Fig 40 Naphthol AS D acetate E 600 10^{-4}M ×480

ELECTROPHORESIS

The results obtained with electrophoresis are summarized in Table 3 and Fig 41 shows the zymograms obtained with different substrates The schematic zymogram in Table 3 is made by pooling the bands obtained with different substrates but actually α naphthyl acetate alone is capable of producing this kind of zymogram In the left hand group are listed the substrates which are hydrolysed in each band showing thus the substrate preference in the right hand group are enumerated the esterases responsible for each band This list is compiled from the inhibitor studies

TABLE 3 *Schematic zymogram illustrating the results obtained with different substrates In the left band group are listed the substrates which are hydrolysed in each band illustrating the substrate preference In the right band group are enumerated the esterases responsible for each band obtained from inhibitor studies*



A = α Naphthyl acetate
B = α Naphthyl butyrate
AS = Naphthol AS D acetate
I = 4-Chloro-5-bromoindoxyl acetate
AcTh = Acetylthiocholine iodide
BuTh = Butyrylthiocholine iodide

Acetylcholinesterase

With acetylthiocholine iodide and 10^{-4}M iso-OMPA (Fig 41 upper row leftmost zymogram) five positive bands were visible corresponding with Bands 5 6 7 9 and 10 of the α naphthyl acetate zymogram in which eserine (10^{-5}M) and 284 C 51 (10^{-5}M) had an inhibiting effect on these bands These same inhibitors totally inactivated the zymogram bands obtained with acetylthiocholine

Non specific Cholinesterase

With butyrylthiocholine iodide and 10^{-5}M 284 C 51 (Fig 41 upper row, the second zymogram from left) one broad zone of activity was obtained the lowest edge of which was more pronounced This area of activity fell between

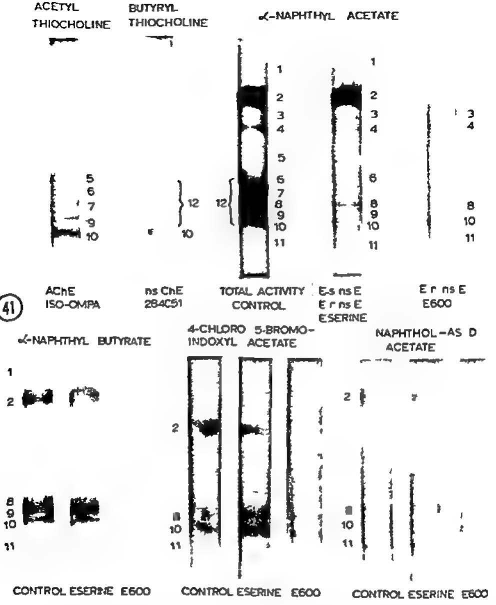


Fig 41 Esterases in zymograms obtained with different substrates and substrate inhibitor combinations Some details present in the original zymograms have been lost due to difficulties in photographic reproduction. The bands have been numbered by comparing with the neighbouring slice developed in α naphthyl acetate to show all the bands

Bands 6—10 in the α naphthyl acetate zymogram and its most active part corresponded to Band 10 *i e* the AChE band which was least mobile In the α naphthyl acetate and 4-chloro-5 bromoindoxyl acetate zymograms there was broad diffuse background staining in this same area which was designated Band 12 for convenience (Fig 41 and Table 3) This broad ns ChE band was inhibited in the butyrylthiocholine zymogram in the presence of 10^{-7}M *iso* OMPA 10^{-5}M *iso* OMPA was required to inhibit Band 12 from the α naphthyl acetate zymogram. Eserine 10^{-5}M inhibited Band 12 in both zymograms

E 600 sensitive Non specific Esterase

With α naphthyl acetate and 10^{-5}M eserine (Fig 41 upper row the second zymogram from right) nine bands were obtained four of which were certainly bands of E-r ns E Thus E-s ns E activity occurred in five bands These were the Bands 1 2, 6 9 and 10 Of these only Bands 1 and 2 showed pure E-s ns E activity Bands 6 and 9 exhibited both ChE and E s ns E activity and in Band 10 all the four types of esterases were demonstrable

In the zymograms obtained with naphthol AS D acetate and 4-chloro-5 bromoindoxyl acetate (Fig 41 lower row) E-s ns E was usually visible only in Band 2, but sometimes with 4-chloro 5-bromoindoxyl acetate E-s ns E occurred in Band 6 Bands obtained with α naphthyl butyrate showed only E-s ns E activity even in Band 10 which with other substrates contained all four types of enzyme activity (*v supra*) In other words the addition of eserine (10^{-5}M) inhibited no component of this zymogram while E 600 (10^{-5}M) inhibited all its constituents The E s ns E bands visualized by α naphthyl butyrate corresponded Bands 1 2 8 9 10 and 11 of the α naphthyl acetate zymogram Band number 11 became visible only when a higher concentration of the starting material than usual was employed

With all the substrates including the thiocholines there was a reaction in the origin this was appreciably weaker after incubation with eserine (10^{-5}M) but the thiocholine reaction was totally inhibited

The initial reaction was abolished by E 600 10^{-5}M This initial reaction was thus due to both ChEs and E s ns E which did not travel along the gel during electrophoresis

E 600 resistant Non specific Esterase

In the presence of E 600 (10^{-5}M) five active bands were observed with α naphthyl acetate (Fig 41 upper row rightmost zymogram) and these corresponded with Bands 3 4 8 10 and 11 of the control α naphthyl acetate zymogram. Bands 3 4 8 and 11 contained E-r ns E only Band 10 instead comprised four kinds of esterase activity

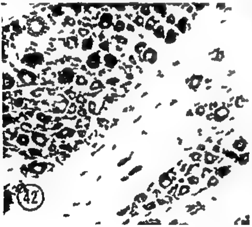
Naphthol AS D acetate and 4-chloro-5 bromoindoxyl acetate produced only three bands corresponding with Bands 8 10 and 11 in the presence of E 600 (10^{-5}M) (Fig 41) As already mentioned α naphthyl butyrate with E 600 differed from the other substrates because it did not give any positive reaction to the zymogram even with prolonged incubation α Naphthyl butyrate did not differ from the other substrates in sections however

Effect of Formalin on the Starch Slab

When a starch slice was incubated in calcium formalin before staining and compared with a serial slice of the same slab preincubated in calcium chloride a slight overall inhibition was observed in the formalin preincubated slab E s ns E Band 2 and the ChE bands were most inhibited by formalin

RIBONUCLEIC ACID

Pyronin stained the ribonucleic acid (RNA) of the ganglion cells intensely red including that in the nucleoli of the ganglion cells The deoxyribonucleic acid in the satellite cell nuclei was intensely stained by methyl green while the nerve cell nuclei stained but poorly The pyronin stain of the ganglion cells was situated in granules which in most of the cells were evenly distributed throughout the cell (Figs 42 and 43) In some cells they were however gathered to the periphery of the cell forming a ring No clear classification of the ganglion cells into »light» and »dark» cells could be made



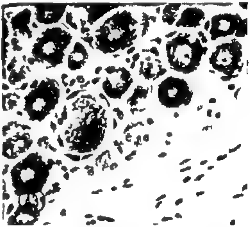


Fig 42 Methyl green pyronin staining in a fixed section × 120
Fig 43 Same section × 240

DISCUSSION

ACETYLCHOLINESTERASE

The most striking observation in the present study was the great variation in intensity of enzyme activity between individual neurones This variation was particularly marked with AChE and it has been commented on by many earlier authors (Giacobini 1956 1959a and b 1960 Gerebtzoff 1959 Tewari and Bourne 1962a and c Cauna and Naik 1963 Strumia and Baima Bollone 1964) However no adequate explanation for this phenomenon has been presented

As a possible explanation for a high activity in some neurones and a low activity in others it can be assumed that the former are cholinergic, the latter non cholinergic ones (Giacobini 1959a and b) Koelle (1954 1955a and b) in fact suggested the existence of a transmitter other than acetylcholine to explain the by him noticed low overall AChE activity in cat and rat spinal ganglia primary sensory neurones of the central nervous system, and the sensory cells themselves It must be acknowledged that also in the present study only a few spinal ganglion cells exhibited a really high AChE activity while most cells reacted but weakly as compared with such cholinergic cells as the motor ventral horn cells of the spinal cord or the cholinergic sympathetic ganglion cells of the superior cervical ganglion of the rat

In this connexion it is of interest that also other sensory cells such as the olfactory sensory cells in mammals and the lateral line cells of fish have been shown to lack AChE in their synapses and cell bodies (Cordier 1964) Esila (1963) found that also the visual cell layer of the retina in all the species examined lacked AChE and only a few species exhibited AChE in outer plexiform layer Thus also these other sensory cells are supposed to be non-cholinergic

It is widely agreed that high concentrations of ACh ChAc and AChE generally correspond well with each others in the cholinergic neurones (Hebb 1963 Koelle 1963 Nachmansohn 1963) Thus the observed low ACh AChE and ChAc activities in the dorsal roots and dorsal horn of the spinal cord give further support for the non-cholinergic nature of most of the sensory nerve cells in spinal ganglia this contrasts with the anterior root and anterior horn synapses known to be cholinergic (Hebb and Krnjevi 1962 Quastel, 1962 Hebb 1963 Koelle 1963) Other transmitters suggested for the spinal ganglion cells include histamine Substance P and adenosine triphosphate (see Crossland 1962)

An alternative explanation for the variation in the AChE intensity from one cell to another might be the presence in addition to rapidly conducting pathways of slow sensory pathways presumably reflected in small amounts of ACh and in low AChE activity of the corresponding neurones Further infrequent stimulation only may occur in visceroceptors minimising the need of AChE in corresponding nerve cell bodies Finally the widely varying length of the dendrites in different cells might be reflected in their AChE activity Assumptions of this kind are purely speculative and have no data for or against them

The significance of the observed tendency of smaller cells to show higher AChE activity will be discussed later when cell size and enzyme activity are correlated

NON SPECIFIC CHOLINESTERASE

Ns ChE was almost exclusively localized in the capillaries and interstitial tissue However occasional neurones also exhibited a weak ns ChE activity The activity in the satellite cells and in the Schwann cells was as a rule intense these two cell types exhibit similar ultrastructural features (see *e g* Pannese 1960) and probably serve similar functions Nutrition and removal of metabolites from nerve cells has long been ascribed to the satellite cells surrounding them (Scharf 1958 Pannese 1960) Metabolic changes might be expected then in the satellite cells which may also serve as a barrier preventing injurious metabolites from reaching the nerve cell (Lajtha 1962) and ns ChE in the satellite cells and capillaries might subserve this function

Tewari and Bourne (1962a) and Cauna and Naik (1963) deny the existence of ns ChE activity in the rat spinal ganglion Most of the earlier studies on the spinal ganglion however support the present observations (Koelle 1951 1955 Coupland and Holmes 1957 Giacobini 1959a and b)

The specific role of ns ChE is still unknown The fact, that ns ChE occurs in blood and almost all tissues might suggest that it hydrolyses choline esters not functionally related to nervous transmission Thus it has been suggested (Clitherow *et al* 1963) that ns ChE might hydrolyse toxic accidental byproducts (mainly butyrylcholine) of fatty acid metabolism in the liver However it is uncertain whether such activity is necessary in the nervous system γ Aminobutyrylcholine has been reported to be present in brain tissue (Kewitz 1959) and may be split by ns ChE (Holmstedt and Sjöqvist, 1960)

The likely explanation that ns ChE assists the hydrolysis of stray ACh near the synapses (Koelle, 1962) does not apply to spinal ganglia since synapses do not exist in them

NON SPECIFIC ESTERASES

As in other publications from this laboratory on nervous tissue (Eränkö *et al* 1964 Härkönen, 1964 Söderholm 1965) the desmo-enzyme nature of E-s ns E and the lyo-enzyme nature of E-r ns E was confirmed in the present study The localizations of these two enzymes were likewise dissimilar in the spinal ganglion

Marked differences in the intensities of E-s ns E and E-r ns E activities in individual cells were found in the present study Differences in total esterase activity of individual nerve cells have earlier been reported in the rat spinal ganglion by Tewari and Bourne (1962a) and in human ganglion cells by Thomas (1963) using naphthol acetates and indoxyl acetate as substrates respectively Since no inhibitors were employed in these studies to exclude AChE and to differentiate the other types of esterases the significance of the observed differences remains unclear

Observations of the present study are compatible with the view that E-s ns E is »cytoplasmic» (a nomenclature used by Novikoff 1961 Shnitka and Seligman

1960 1961 Wachstein and Meisel 1960 in contradistinction to »lysosomal bound» esterase) and attached to endoplasmic reticulum F r ns E is probably mainly attached to cytoplasmic granules This enzyme localization has both biochemical (Aldridge and Johnson 1959) and electron microscopical support (Torack and Barrnett 1962) In the latter study ns F was located both in the endoplasmic reticulum and in »dense bodies» The fact that F r ns E was in the present work observed exclusively in cytoplasmic granules lead to a further study of the properties of these granules (see page 39)

Non specific esterase activity can be found in nearly all nerve cells (Pepler and Pearse 1957 Felgenhauer 1964) The ns E of the cerebral cortex is mainly E-s ns E (Pepler and Pearse 1957) while many brain nuclei the hypothalamus and the ependyma contain L-r ns F activity suggested to be due to cathepsin (Pepler and Pearse 1957 Felgenhauer 1964) However the function of either E s ns E or E r ns F in the nervous tissue is still totally unknown

Elsewhere in the organism notably in the liver and the kidney an abundance of esterases has been found (Gomori 1955) Although the distribution of these enzymes has been widely studied by histochemists no final explanation for their significance for animal organism has been advanced To illustrate the much varying views presented some examples will be given Many authors consider that ns E influences growth and development (Mendel *et al* 1953 Smith and Wagenknecht 1959) Hopsu and Glenner (1963 1964) and Hopsu *et al* (1965) have postulated a proteolytic function for ns E activity Novikoff (1961) on the other hand has suggested that lysosome bound E r ns F can act as a lipase Catabolic and anabolic functions for this enzyme group are not mutually exclusive and they might thus be related to general cell metabolism None of these hypotheses can furnish an adequate explanation for the ns E activity in the nervous tissue

ELECTROPHORESIS

The present observations are in agreement with the earlier reports as to the multiplicity of esterases separated by electrophoresis as demonstrated with several substrate inhibitor combinations (Markert and Hunter 1959 Eränko *et al* 1962a and b 1964 Harkonen 1964 Soderholm 1965) The properties of the (highly soluble) lyo-esterases and of the desmo-esterases (firmly attached to tissue) have also been discussed earlier (Eranko *et al* 1964 Harkonen, 1964 and Söderholm 1965) These aspects of the electrophoresis of esterases will therefore not be further dealt with in the present paper

Bands were observed in the zymogram of the spinal ganglion in each of which more than one type of esterase activity was present Thus both AChE and E s ns E were detected in Band 9 and these two enzymes were in addition to one or two other types of esterase activity also found at the origin and in Bands 6 and 10 Similar findings have previously been reported in nervous tissue (Harkönen, 1964 Soderholm 1965) Four theories can be advanced to explain bands with more than one type of enzyme activity (1) the band is formed by several separate enzymes which have the same electrophoretic mobility (2) more than one enzyme is attached to a carrier protein which moves along with the enzymes

in the starch gel (3) an enzyme protein is of a multi locus type containing diffe rent active sites responsible for enzymatic hydrolysis of different esters and (4) one and the same enzyme is capable of hydrolysing different substrates even in the presence of selective inhibitors

The attachment of several enzymes into a carrier protein may in the present case be the most likely one Apart from the first possibility (*i e* accidental simi larity in the electrophoretic mobilities of several esterases) all the other hypotheses would indicate the presence of several types of esterase activity in the same cyto- plasmic organelle of a cell unless different esterases acquire an attachment to the same protein during homogenization

If for the above reasons two or more types of esterase activity are present in the same cell due to association of the corresponding enzymes with one protein one would expect a tendency towards correlation both in the activity levels and in the cytological locations of two different types of esterase activity These possibilities will be be returned to later (page 68)

RIBONUCLEIC ACID

The methyl green pyronin staining was carried out to compare the distribu tion of enzymes with that of RNA In contradiction to many previous authors (see Scharf 1958) it was not possible to classify the cells into »dark» and »light» ones as judged by the intensity of RNA staining with pyronin all the cells being fairly equally stained Occasional cells seemed darker than their neighbours but such cells had shrunk and lost contact with their capsules This was also true of paraffin-embedded material The author is therefore inclined to agree with Fisher and Ranson (1934) that fixation artefact is the explanation for most of the dark cells previously reported

The problem of dark and light cells has also interested many electron micros copists but their results are also contradictory probably due to different methods of fixation and embedding (Hess 1955 Cervós Navarro 1959 Pannese 1960 Andres 1961 Smith 1961)

II ACID PHOSPHATASE

LITERATURE REVIEW

Acid Phosphatase in the Spinal Ganglia

The distribution of acid phosphatase (AcPh) in the spinal ganglion of the rat has been described by Colmant (1959) who used both an azo-coupling and a lead method He described two types of cells large weakly reacting cells and small strongly positive ones He also observed intermediate forms Division of the sciatic nerve increased activity of the light cells while the reverse was true of the dark cells Satellite and Schwann cells showed no activity

Tewari and Bourne (1962a) who used an azo-coupling method similarly reported that the small cells in the rat spinal ganglion exhibit a more intense AcPh activity than the large cells The enzyme activity was reported to be localized in granules Similar findings have also been described (Galabov *et al* 1964) in the rabbit spinal ganglion with a lead technique

Intracellular Localization

Neuronal acid phospatase has been localized both with the electron and light microscope in dense bodies probably lysosomes in some parts of the Golgi apparatus and to a lesser degree in the endoplasmic reticulum (Novikoff and Essner 1962 Novikoff 1963 Osinchak, 1963 Smith 1963 and Sharma 1964) Ribosome dependent manufacture of acid phosphatase has been suggested to be followed by the transport of the enzyme to the Golgi saccules and vesicles and thence to the dense bodies or lysosomes (Goldfischer *et al* 1964 Novikoff *et al* 1964) Lipofuscin granules have also been reported to show acid phosphatase activity (Gedigk and Bontke 1956 Koenig *et al* 1963 Koenig 1964 Keefe and Ordy 1964) Anderson and Song (1962) feel that this is not the case in neurones with large amounts of pigment, believing that the acid phosphatase reaction is confined to the periphery of the pigmented area

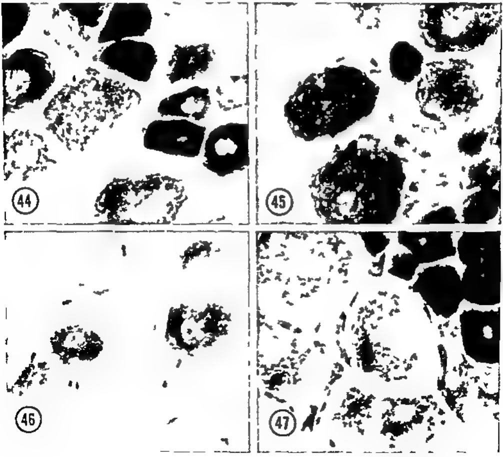
METHOD

Incubation solution for acid phosphatase was made according to Gomori (1952) with a mixture of equal parts of sodium α and β glycerophosphate as substrate Both fresh and fixed sections were incubated for 30—45 mins at 37 C After incubation the section was treated with dilute yellow ammonium sulphide

Some experiments were carried out with azo-coupling techniques Since the results obtained with them were less satisfactory than those with the lead method further studies were abandoned

RESULTS

In fresh sections all ganglion cells exhibited activity but great variations were observed in intensity in different cells (Figs 44 and 45) The reaction was always most intense in small cells but some small cells exhibited a weak reaction Occasio



Figs 44 and 45 Acid phosphatase in fresh sections ×480

Figs 46 and 4 Acid phosphatase in fixed sections × 480 Note the difference in the cytoplasmic distribution of the reaction as compared with *Figs 44* and *45*

nal large cells showed a relatively intense reaction but the largest cells were never as reactive as most of the small ones

In most of the cells the precipitate was diffuse but particularly in some larger cells the reaction product appeared as a network (Fig 45) Nuclei and nucleoli were always negative Some satellite cells were positive but as a rule weaker than the ganglion cells which they surrounded Around some nerve fibres a positive reaction was seen perhaps in the Schwann cells

In fixed sections the reaction product was always localized exclusively in cytoplasmic granules In some of the cells these granules were dispersed all over the cell (Fig 46) Perinuclear and peripheral accumulations occurred (Fig 47) Some of the small cells were full of active granules (Fig 47) Variation in the intensity of acid phosphatase activity of individual cells exceeded that found in fresh sections The intense perinuclear reaction in the satellite cells forming a mantle round unreactive neurones was particularly striking in fixed sections If overincubated (2 hours) also some nerve fibres showed a reaction The granular distribution of acid phosphatase thus closely resembled the distribution of E-r ns E This similarity created the question whether these two types of enzyme activity are located in the same granules This problem is examined in Chapter III of the present paper

DISCUSSION

An interesting feature observed in the present work was the difference in the distribution of acid phosphatase activity between fresh and fixed sections This difference suggests the existence of soluble and particle bound forms of acid phosphatase whose cytoplasmic locations are different Lison (1948) early pointed out the possibility of lyo- and desmo-phosphatases and Nachlas *et al* (1956) have indeed shown that in fresh sections a part of the acid phosphatase activity is easily lost in water or buffer

If acid phosphatase during life was present exclusively in cytoplasmic granules including lysosomes sectioning and handling of fresh material seems sure to disrupt some of these spilling their contents and this in part may account for the diffuse type of reaction seen in unfixed sections (Bitensky 1963 De Duve 1963 Tappel *et al* 1963 Sawant *et al* 1964) Tappel *et al* (1963) are however of the opinion that the free cytoplasmic acid phosphatase really differs from its granule bound counterpart although the greatest part of free acid phosphatase would presumably come from lysosomes

Degenerative changes were not observed in those cells which exhibited an intense acid phosphatase activity This must be clearly stated since it has been supposed that a high acid phosphatase activity is often associated with cell degeneration (Bitensky 1963 Novikoff 1961)

It is of interest that a high acid phosphatase activity has been found in endocrine secretory cells in the choroid plexus and in neurosecretory cells (Eranko 1951 Pearse 1960 Novikoff 1961) suggesting that acid phosphatase may be involved with active protein synthesis Continual protein synthesis is a feature of all nerve cells the completed products moving from the perikaryon to the

periphery along the axon (Singer 1964) A similar kind of flow may also occur in the dendrites of sensory nerve cells Differences in the activity of acid phosphatase may thus perhaps reflect the rate of protein synthesis in individual neurones, which in turn may depend on the length of their processes

Since small cells exhibited higher enzyme activity than large cells it seemed appropriate to study the possible relation of enzyme activity to cell size This aspect is covered in Chapter IV of the present paper

III CYTOPLASMIC GRANULES IN NERVE CELLS

LITERATURE REVIEW

Besides mitochondria there are other cytoplasmic granules in nerve cells whose nature has interested many authors These granules have been approached from various points of view (using *e g* classical histological methods and histo chemical methods both at the light and electron microscopical level) This has lead to a large variety of names given to the granules Abnutzungspigment wear and tear pigment lipofuscin fluorescent granules pigment bodies dense bodies lysosomes residual bodies A comparable variety of functions has been ascribed to them It is quite evident that though different types of granules exist several names have been applied to the same granule and the interrelation ship of different inclusions is unclear The features of the various granules will now be summarized

Unstained human nerve cells contain yellow brown granules as was noted as early as in the end of the 19th century (Hodge 1894/95 Pilcz 1895) This yellow pigment has been termed Abnutzungspigment or lipofuscin It occurs in other primates and in small amounts in the nervous tissues of old guinea pigs horses cows and dogs The intensity of the colour of the pigment varies from one species to another but most authors agree that the colour is deepest in human nerve cells (see Scharf 1958) In man the yellow pigment is always present from the seventh year of life and occasionally already at birth (Muhlman 1901 Stohr 1941 Hermann 1951) Thus it can hardly be regarded as a sign of senility and some authors have ascribed to it a metabolic or storage function (Muhlman 1901 Stohr 1941)

In summarizing the characteristics of lipofuscin Pearse (1960) states that it is weakly PAS positive strongly basophilic stains weakly with Sudan Black B and exhibits an autofluorescence

Hamperl (1934) found in man however fluorescent granules not only in nerve cells but also free in the neuropil and in the glial cells Moreover fluores cent granules were widely found in non nervous organs such as the liver the thyroid the testis the ductus epididymidis the prostate and the vesicula seminalis

In the nerve cells moreover Hamperl (1934) observed fluorescent granules which unlike the fluorescent lipofuscin granules were devoid of pigment Sjostrand (1944) in a study of autofluorescent granules of various organs in rats mice guinea pigs and rabbits confirmed Hamperl s findings and stressed that the number of fluorescent granules is greater than that of visible pigment

granules. Cells entirely devoid of pigment occurred in which there were numerous fluorescent granules.

Björkerud and Zelander (1960) isolated the yellow pigment of human nerve cells (spinal medulla) and studied its autofluorescence and ultrastructure. In spinal ganglia osmiophilic pigment granules assumed to be lipofuscin granules have been described although full data regarding their fluorescence and natural colour is lacking (Hess 1955, Cervos Navarro 1959, Andres 1961, Smith 1961, Keefe and Ordy 1964).

Koenig has added acid phosphatase, deoxyribonuclease, ribonuclease and esterase activity to Pearse's (1960) criteria for lipofuscin pigment. Koenig initially regarded lipofuscin and lysosomes as identical but later reported that true lipofuscin granules contain less acid phosphatase. The fluorescent constituents of lysosomes and lipofuscin may be associated with a glycolipoprotein matrix and lipofuscin may be derived from lysosomes (Koenig 1962, 1963a and b, 1964, Koenig *et al.* 1963).

Alternative views on the origin of lipofuscin granules exist. Hess (1955) with the aid of electron microscope implicated mitochondria in the formation of pigment bodies and Tewari and Bourne (1962b) reached a similar conclusion based on their as yet unconfirmed observation on similar distributions of β glucuronidase and succinic dehydrogenase in nerve cells.

A further origin for lipofuscin pigment has been proposed by Issidorides and Shanklin (1961) and by Sharma (1962) who consider it to be a neurosecretory product. The former authors postulated a relationship between lipofuscin pigment and Nissl bodies and they describe abundant extracellular lipofuscin granules in the synaptic areas surrounding Purkinje cells. This extracellular localization of cerebellar lipofuscin pigment is in accordance with the findings of Stammler (1959) who observed that the histochemical behaviour of the extracellular granules corresponds with that of the intracellular pigment deposits although the former were not coloured.

Lastly, acid phosphatase and esterase activity has been localized in lipofuscin granules by several authors including Gomori (1955), Pearse (1955), Gedigk and Bontke (1956), Anderson and Song (1962), Jamieson (1963), Keefe and Ordy (1964) and Samorajski *et al.* (1964).

METHODS

AUTOFLUORESCENCE

Autofluorescence was studied in fresh sections dried on a slide and in fixed sections mounted in glycerol. Light from an Osram HBO 200 high pressure mercury lamp was filtered through two Schott K2 heat absorbing filters, Schott BG [illegible] ultraviolet filter and BG 23 red absorbing filter. Schott OG 1 served as a fluorescence filter. A rapid film was used in fluorescence photography so as to reduce the exposure of the section to a minimum thus avoiding inhibition by ultraviolet light of the enzyme to be subsequently demonstrated.

HISTOCHEMICAL STAINING METHODS

Autofluorescent granules were first photographed in a section and this was subsequently stained

The periodic acid Schiff (PAS) reaction was studied as given by Pearse (1960) Staining with Sudan Black B was performed according to Baker s (1944) supersaturation technique (quoted by Lillie 1947) E r ns F reaction was carried out as described in Chapter I Because only formalin fixed sections exhibited a purely granular acid phosphatase reaction this comparison with fluorescence was also made on fixed sections using the method described in Chapter II In addition, the distribution of fluorescence according to Koenig s (1963a) modification of Gomori s method was examined After incubation the residual fluorescence was first photographed in ultraviolet light Then the section was either inspected with dark field illumination and the refractile lead phosphate precipitates photographed or the section was treated with ammonium sulphide in the usual way and the brown lead sulphide deposit photographed

RESULTS

NATURAL COLOUR OF THE GANGLION CELLS

In unstained fresh or fixed spinal ganglia of old rats (over $1^1/_2$ years) eccentric aggregations of pale yellow granules were seen in some ganglion cells Coloured granules were never observed in the spinal ganglia of young rats The yellow granular clumps of old ganglion cells were better demonstrated at a wavelength of 4360 Å the contrast increasing due to more intense absorption of blue light by the yellow pigment In spinal ganglion cells of young rats no absorbing granules were seen even at this wavelength

AUTOFLUORESCENCE

When unstained fresh or formalin fixed sections were viewed in ultraviolet light, granules exhibiting a yellow autofluorescence were seen in most of the ganglion cells and in some of the satellite cells (Fig 48) In some ganglion cells these granules were situated around the nucleus forming a broad ring In some other cells they formed an aggregate at one side of the cell. However as a rule the granules were evenly dispersed throughout the cell This distribution of fluorescent granules strikingly resembled that of E r ns E and acid phosphatase as described earlier in this paper

LIPIDS, POLYSACCHARIDES AND FLUORESCENCE

When the autofluorescent granules were first photographed in a section and this was subsequently coloured with Sudan Black B many fluorescent granules were thus found sudanophilic. However fluorescent granules were always more numerous than the sudanophilic granules Moreover this was the case also with PAS positive granules some of them were identical with fluorescent granules and these tended to aggregate peripherally but the number of fluorescent granules always exceeded that of the PAS positive granules

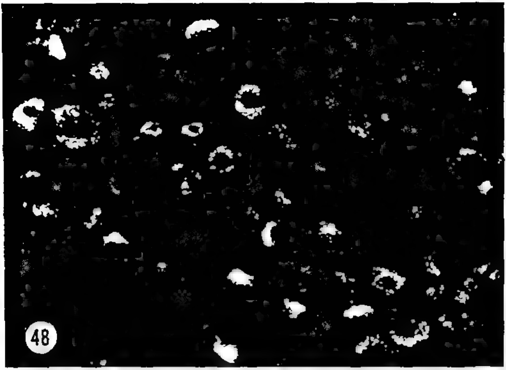


Fig 48 Autofluorescence in a fixed section. × 225

E 600 RESISTANT NON SPECIFIC ESTERASE AND FLUORESCENCE

When the fluorescence was photographed and the same section was then treated to demonstrate E r ns E it was found that the fluorescence and the E r ns E pictures were closely similar Strongly fluorescent granules often were intensely E-r ns E-positive (Cell type 1 labelled with arrows 1 in Figs 49 and 50) and many cells exhibiting no fluorescence were devoid of E r ns E activity (Cell type 2 labelled with arrows 2 in Figs 49 and 50)

Careful and detailed study of individual granules revealed the following exceptions to the above rules firstly some strongly fluorescent cells contained but few E-r ns E positive granules (Cell type 3 labelled with arrows 3 in Figs 49 and 50) Secondly poorly fluorescent cells occurred showing numerous granules of an intense E r ns E activity (Cell type 4 labelled with arrows 4 in Figs 49 and 50)

Adjacent to Figs 49 and 50 are displayed several pairs of cells extracted from Figs 49 and 50 and from other similar pairs of pictures The pairs of cells are arranged so as to illustrate the four described cell types Figs 51 and 52 show a part of the same section as in Figs 49 and 50 at a higher magnification

No attempts were made to estimate accurately the relative frequencies of these four types of cells There were always more fluorescent granules than E r ns E positive granules and the fluorescent granular aggregations matched well the aggregations of E r ns E granules

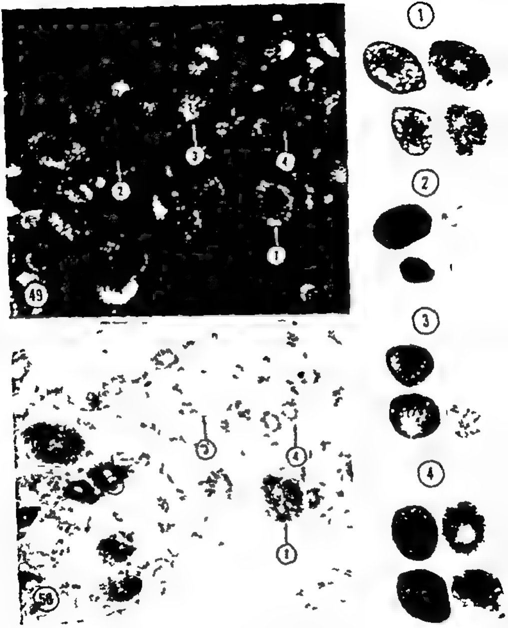


Fig. 49 Autofluorescence in a fixed section × 240
Fig 50 E r ns E in the same section × 240

Examples of the four cell types described in the text are indicated by arrows. On the right from *Figs 49* and *50* cut-out pairs of photomicrographs of individual cells of the four types In each pair the left one is of fluorescence the right one of E r ns.E in the same cell.

Fig 51 Autofluorescence in a fixed section × 375
Fig 52 E r ns E in the same section × 375

ACID PHOSPHATASE AND FLUORESCENCE

By photographing the fluorescence first and then demonstrating the acid phosphatase activity by usual way (Figs 53 and 54) it was found that fluorescent and acid phosphatase positive granules were not as well correlated as fluorescent and E r ns E granules Fluorescent granules in the satellite cells however seemed usually to be identical with the acid phosphatase positive granules

In ganglion cells again four types could be recognised (1) cells with intensely fluorescent granules which were acid phosphatase positive (2) cells exhibiting *no fluorescence and no acid phosphatase activity*, (3) *strongly fluorescent cells* which were essentially acid phosphatase negative (4) cells showing an intense acid phosphatase reaction but little or no fluorescence On the whole there were more fluorescent granules than acid phosphatase positive granules

In sections prepared with Koenig s method the positive correlation between the fluorescence after incubation and the histochemical acid phosphatase reaction was nearly absolute (Figs 55 and 56) However this method is open to criticism and of limited value in the present case since the fluorescence in a section thus incubated differed materially from a non incubated section This applies especially to cells which were intensely acid phosphatase positive and which in a fresh or fixed section exhibit little or no fluorescence

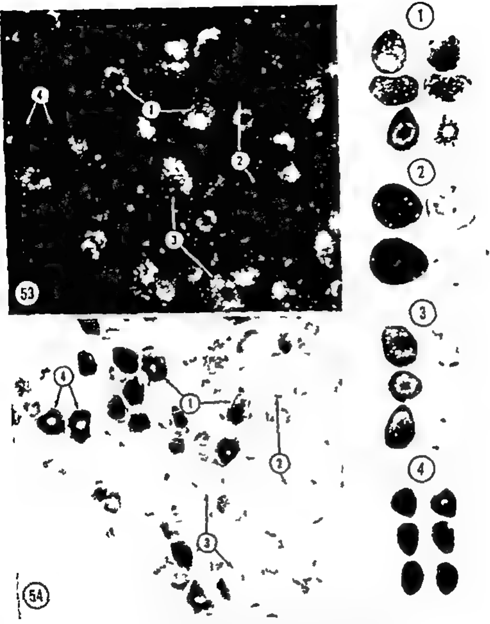


Fig. 53 Autofluorescence in a fixed section, × 240

Fig 54 Acid phosphatase in the same section × 240

Examples of the four cell types described in the text are indicated by arrows On the right from *Figs 53* and *54* cut-out pairs of photomicrographs of individual cells of the four types In each pair the left one is of fluorescence the right one of acid phosphatase in the same cell.

Fig 55 Fluorescence in a section incubated in Gomori s medium for acid phosphatase ×300 Note that the fluorescence differs from that in *Figs 48 49 51* and *53*

Fig 56 The same section after treatment with ammonium sulphide × 300

DISCUSSION

In the present study only the granules of old animals exhibited an intrinsic colour Since colour is by definition the main property of any pigment it is not justified to use the term pigment unless colour is seen Thus no pigment is present in rats younger than one year and very little even in older ones However since lack of pigmentation again is the main feature of albinos granules corresponding with lipofuscin of pigmented animals can be expected to be colourless although they would retain other properties of these granules

Samorajski *et al* (1964) have demonstrated that pigmented autofluorescent lipofuscin in the neurones of old men hooded rats and mice has the same distribution as lipid staining with Sudan the PAS reaction as well as acid phosphatase and cathepsin C type esterase activity Except for the lack of colour in the fluorescent granules the present findings are in many features similar However in the present study the number of fluorescent granules exceeded the number of granules exhibiting enzyme activity This was true concerning both acid phosphatase and E r ns E activity

While Samorajski *et al* thus considered acid phosphatase acid esterase PAS and Sudan positive granules to be mainly lipofuscin granules Sharma *et al* (1965) in a study of the cells of rat spinal cord considered granules with similar histochemical properties to be lysosomes

While this may be mainly a matter of terminology the fact that in the present study the granules possessing acid phosphatase E r ns E activity and fluorescence tend to gather in peripheral aggregations could be interpreted as support for the theory that the lysosomes may gradually alter into lipofuscin granules (Essner and Novikoff 1960 Koenig 1963a Samorajski *et al* 1964)

The fluorescence which is characteristic of both lipofuscin and lysosomes is according to Koenig (1962, 1963a and b 1964) due to a glyco-lipoprotein matrix. On the other hand Tappel *et al* (1963) stated that flavin fluorescence is characteristic of lysosomal fluorescence and that this differs from mitochondrial fluorescence

De Duve who first proposed the term lysosome, does not strictly define it (1963) but instead acknowledges lysosomes to be heterogeneous in their enzymic characteristics Like many other authors (*e g* Bitensky 1963 Tappel *et al* 1963 Sawant *et al* 1964) he emphasizes that the lysosomes of various organs may differ not only in enzyme content but also in others respects (*e g* fragility) The lysosome concept is thus rather loosely defined

In the present study all the fluorescent granules were not enzymatically active and on the other hand all the enzymatically active granules were not fluorescent. Apart from acid phosphatase and E-r ns E, this seemed to be the case also concerning β glucuronidase and leucin amino-peptidase (LAP) activity both of which are generally assumed to be lysosomal enzymes β Glucuronidase was found only in the cytoplasmic granules of the smaller cells and it was not possible to demonstrate any LAP in any of the nerve cells (unpublished observation)

If the loose definition of the lysosome-concept is accepted all the cytoplasmic granules of the present study could be interpreted as lysosomes Granules devoid of enzyme activity might be somehow exhausted or they might contain other lysosomal enzymes not demonstrated here. Until the presence or absence of several enzymes in a given granule can be determined the problem of the heterogeneity of the granules remains speculative and the terminological argument cannot be resolved

IV COMPARISON OF THE ENZYME ACTIVITIES AND THE SIZE OF INDIVIDUAL CELLS

In the present work both acetylcholinesterase and acid phosphatase activity seem to be higher in smaller cells than in larger ones and similar conclusions have been reported by earlier workers (Colmant 1959 Tewari and Bourne 1962a Galabov *et al* 1964) However no quantitative cytophotometric data have been presented in this connection to date

Since the histochemical variations were observed by the present writer in the activity of individual ganglion cells with all hydrolytic enzymes examined, the question arose whether there is any positive or negative correlation between the enzyme activity levels and cell size in individual cells

METHODS

MEASUREMENT OF THE CELL DIAMETER

The diameter of the cell fragment present in the section was measured from photomicrographs taken at a linear magnification of 200 × The measurement was carried out to the nearest mm an arbitrarily selected plane was used for measuring all cells in the same photomicrograph. Since there was only a fragment of each cell in a section some tangential caps of large cells have been regarded as small cells and the cell diameters obtained tend to be too small. In those cases in which two different enzymes were studied in neighbouring sections of the same cell, the larger one of the two diameters thus obtained was therefore used for calculations Since the inaccuracy of the diameter estimation mentioned increases the scatter this error results in an artificially low correlation coefficient, when the diameter of the cell is compared with the enzyme activity

DEMONSTRATION OF TWO TYPES OF ENZYME ACTIVITY IN INDIVIDUAL CELLS

Initially two different enzymes were consecutively stained in a single section. However although this proved possible it was observed that the reaction first applied always partially inhibited the second one tending to give false negative correlations between the two types of enzyme activities examined. Therefore two serial sections of 5 μ of thickness were separately treated to demonstrate different enzymes Since the ns ChE activity is negligible in all ganglion cells as compared with the AChE activity the sections which were used to the estimation of AChE activity were processed without inhibitor using acetylthiocholine as substrate Thus the ns ChE positive satellite cells became visible which greatly facilitated the identification of AChE negative cells otherwise difficult to recognise as cells (Figs 61 and 68) After photomicrographs the same cells were identified in the pairs of pictures thus obtained and numbered in both photographs (Figs 61 and 62 68 and 69)

VISUAL ESTIMATION OF ENZYME ACTIVITY

The intensity of each reaction was subjectively estimated in the numbered cells by examining the corresponding section under the microscope. The intensity was judged as negative, weak, moderate or strong. Subjective bias as a source of error was minimised by judging the reaction in each cell without knowing the results of the other reactions.

PHOTOMETRIC MEASUREMENT OF ENZYME ACTIVITY

The photometer consisted of a Leitz Ortholux microscope with the standard incandescent lamp, supplied with a 5 A current. The magnified image of a small area of the section was projected on the measuring plane with the aid of a 40 × objective and 10 × ocular at a linear magnification of 550. In the image plane a round aperture 2 mm in diameter limited the field to be measured, corresponding to a diameter 3.6 μ in the plane of the section. Above the aperture there was a diffusing ground glass and above it an RCA 1 P 28 photomultiplier tube connected with a battery voltage source and a galvanometer whose sensitivity could be adjusted with a potentiometer. A cobalt glass filter or a blue green filter together with $CuSO_4$ cuvette provided a suitable density range for the measurements. The density was measured by setting the galvanometer at 100 % transmission when an area of the slide free of tissue was under the microscope, then the cell to be measured was moved into the field and a new transmission reading made.

Three transmission readings were taken from the cytoplasm of each cell measured. These three transmission values were converted into terms of density and the mean of these three density values was used to indicate the reaction intensity. Similar measurements on the same cells were carried out in three subsequent days in order to obtain an idea of both the error of sampling and that of optical measurement. Visual selection of the areas to be measured was necessary to be sure that the numbered cell was involved. The danger of subjective bias was minimised by making the measurements without any knowledge of the earlier measurements or the results of visual estimation.

RESULTS

QUALITATIVE OBSERVATIONS

Acetylcholinesterase and Non specific Cholinesterase

Although there were few ns ChE positive ganglion cells, always when a ganglion cell exhibited ns ChE activity it exhibited also an intense AChE activity.

Acetylcholinesterase and Ribonucleic Acid

While AChE activity always varied much from one cell to another, very little such variation was observed in the intensity of the pyronin staining (Figs. 57 and 58). Some coarse Nissl bodies appeared to coincide with the AChE positive granules but, as a rule, the intensity of the AChE reaction did not match that observed of the RNA stain.

E 600-resistant Non specific Esterase and Ribonucleic Acid

Neither the distribution nor the intensity of the E r ns E reaction resembled those of the pyronin stain (Figs. 59 and 60). Moreover, in some cells in which

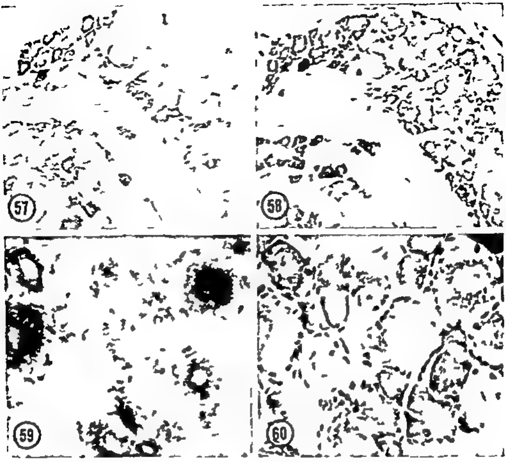


Fig 57 Cholinesterases in a fixed section. Acetylthiocholine no inhibitor × 120
Fig 58 The same section, stained with methyl-green-pyronin × 120
Fig 59 E-r ns.E in a fixed section. α Naphthyl acetate E 600 10^{-4}M. × 240
Fig 60 The same section, stained with methyl-green pyronin × 240

E-r ns E was aggregated at the periphery of the cell, Nissl substance was lacking from this area. However cells with E-r ns E all over the cell were never totally devoid of the Nissl substance.

Non specific Cholinesterase and Cell Diameter

Only a few ganglion cells in fresh or fixed sections exhibited ns ChE activity, and even when present, it was in any case weak. The majority of positive cells were small, and thus there was a kind of negative correlation between the cell diameter and the enzyme activity

Ribonucleic Acid and Cell Diameter

Because all the ganglion cells were about equally stained with pyronin both in fresh and in fixed sections correlation of the cell size and the RNA content did not seem feasible

QUANTITATIVE OBSERVATIONS

Visual Estimations

Visually Estimated Enzyme Activities and the Cell Diameter

Tables 4—9 present correlations between the cell diameter and visually estimated intensity of the activity of acetylcholinesterase E 600-sensitive non specific esterase E 600-resistant non specific esterase and acid phosphatase respectively The results are summarized in Table 10 From these tables it can be seen that the intensities of acetylcholinesterase and acid phosphatase activities bear a significant negative correlation to the cell diameter both in fresh and fixed sections while non specific esterases are not correlated with the cell diameter

TABLE 4 *Correlation of visually estimated intensity of acetylcholinesterase activity (y) and the cell diameter (x) in fresh sections The number of cells falling into each category is given*

Cell diameter (mm)[1] / AChE activity (arbitrary units)	2	3	4	5	6	7	8	9	10	11	Sum
Strong (3)	1	4	10	8	5	4	2	0	1	0	35
Moderate (2)	0	0	5	13	18	10	2	[illegible]	7	1	65
Weak (1)	0	2	7	[illegible]	13	6	7	[illegible]	5	0	50
Negative (0)	0	1	2	1	3	7	4	0	3	2	23
Sum	1	7	24	30	39	27	15	11	16	3	173

[1] Measured from photographs taken at a magnification of 200 × Real diameters in microns can be obtained by multiplying with 5

Mean AChE activity = 1 65
Mean diameter = 6 38
Regression equation $y = -0\,12x + 2\,43$

Correlation coefficient $r = -0\,26$
Residual standard deviation = 0 92
Significance of correlation $P < 0\,001$

TABLE 5 *Correlation of visually estimated intensity of acetylcholinesterase activity (y) and the cell diameter (x) in fixed sections The number of cells falling into each cathegory is given*

Cell diameter (mm)[1] / AChE activity (arbitrary units)	3	4	5	6	7	8	9	10	11	Sum
Strong (3)	1	6	6	2	2	2	0	0	0	19
Moderate (2)	4	4	6	9	5	0	2	0	0	30
Weak (1)	3	4	5	1	6	5	2	0	0	26
Negative (0)	0	5	3	2	2	5	3	2	3	25
Sum	8	19	20	14	15	12	7	2	3	100

[1] Measured from photographs taken at a magnification of 200 × Real diameters in microns can be obtained by multiplying with 5

Mean AChE activity $= 1.43$
Mean diameter $= 6.01$
Regression equation $y = -0.19x + 2.57$
Correlation coefficient $r = -0.36$
Residual standard deviation $= 1.0$
Significance of correlation $P < 0.001$

TABLE 6 *Correlation of visually estimated intensity of E 600-sensitive non specific esterase activity (y) and the cell diameter (x) in fresh sections The number of cells falling into each cathegory is given*

Cell diameter (mm)[1] / E-s ns E activity (arbitrary units)	3	4	5	6	7	8	9	10	11	12	Sum
Strong (3)	6	7	5	5	1	2	1	0	0	0	27
Moderate (2)	11	7	15	18	11	14	12	6	2	1	97
Weak (1)	12	8	3	8	2	2	5	4	4	1	49
Negative (0)	0	0	0	0	0	0	0	0	0	0	0
Sum	29	22	23	31	14	18	18	10	6	2	173

[1] Measured from photographs taken at a magnification of 200 × Real diameters in microns can be obtained by multiplying with 5

Mean E s ns E activity $= 1.87$
Mean diameter $= 6.18$
Regression equation $y = -0.03x + 2.05$
Correlation coefficient $r = -0.11$
Residual standard deviation $= 0.66$
Significance of correlation $P > 0.1$

TABLE 7 *Correlation of visually estimated intensity of E 600-resistant non specific esterase activity (y) and the cell diameter (x) in fixed sections The number of cells falling into each cathegory is given*

Cell diameter (mm)[1] / E r ns E activity (arbitrary units)	3	4	5	6	7	8	9	10	11	12	Sum
Strong (3)	2	5	6	5	7	6	4	1	0	1	37
Moderate (2)	3	4	5	6	4	7	4	1	0	0	34
Weak (1)	3	3	5	2	3	2	1	1	0	0	20
Negative (0)	1	2	0	2	0	1	1	1	1	0	9
Sum	9	14	16	15	14	16	10	4	1	1	100

[1] Measured from photographs taken at a magnification of 200 × Real diameters in microns can be obtained by multiplying with 5

Mean E r ns E activity $= 1\,99$
Mean diameter $= 6\,32$
Regression equation $y = 0\,03x + 1\,83$

Correlation coefficient $r = 0\,06$
Residual standard deviation $= 0\,97$
Significance of correlation $P > 0\,1$

TABLE 8 *Correlation of visually estimated intensity of acid phosphatase activity (y) and the cell diameter (x) in fresh sections The number of cells falling into each cathegory is given*

Cell diameter (mm)[4] / AcPh activity (arbitrary units)	3	4	5	6	7	8	9	10	11	Sum
Strong (3)	5	18	31	11	6	0	1	0	0	72
Moderate (2)	9	23	20	29	18	17	8	2	0	126
Weak (1)	5	20	22	24	12	16	2	3	1	105
Negative (0)	0	0	0	0	0	0	0	0	0	0
Sum	19	61	73	64	36	33	11	5	1	303

Measured from photographs taken at a magnification of 200 × Real diameters in microns can be obtained by multiplying with 5

Mean AcPh activity $= 1\,89$
Mean diameter $= 5\,70$
Regression equation $y = -0\,09x + 2.39$

Correlation coefficient $r = -0\,19$
Residual standard deviation $= 0\,75$
Significance of correlation $P < 0\,001$

TABLE 9 *Correlation of visually estimated intensity of acid phosphatase activity (y) and the cell diameter (x) in fixed sections The number of cells falling into each category is given*

AcPh activity (arbitrary units) \ Cell diameter (mm)[1]	4	5	6	7	8	9	10	11	12	Sum
Strong (3)	9	23	24	7	5	2	0	0	0	70
Moderate (2)	8	14	13	20	30	9	12	5	1	112
Weak (1)	2	8	5	12	8	4	6	4	1	50
Negative (0)	0	0	0	0	1	1	0	1	0	3
Sum	19	45	42	39	44	16	18	10	2	235

[1] Measured from photographs taken at a magnification of 200 × Real diameters in microns can be obtained by multiplying with 5

Mean AcPh activity = 2.06
Mean diameter = 6.96
Regression equation $y = -0.15x + 3.10$

Correlation coefficient $r = -0.38$
Residual standard deviation = 0.70
Significance of correlation $P < 0.001$

TABLE 10 *Correlation of visually estimated intensities of enzyme activities (y) and the cell diameter (x)*

Enzyme	Number of cells studied (n)	Mean intensity of enzyme activity (y) (arbitrary units)	Mean diameter (mm) (x)	Correlation coefficient (r)	Significance of correlation
AChE (fresh sections)	173	1.7	6.4	−0.26	$P < 0.001$
AChE (fixed sections)	100	1.4	6.0	−0.36	$P < 0.001$
E-s ns E (fresh sections)	173	1.9	6.2	−0.11	not significant
E-r ns E (fixed sections)	100	2.0	6.3	0.06	not significant
AcPh (fresh sections)	303	1.9	5.7	−0.19	$P < 0.001$
AcPh (fixed sections)	235	2.1	7.0	−0.38	$P < 0.001$

Intercorrelations of Visually Estimated Enzyme Activities

Results of the comparison of acetylcholinesterase and E 600-sensitive non specific esterase activity in individual cells are presented in Table 11 Figs 61 and 62 show neighbouring sections in which the above mentioned enzyme activities are demonstrated some cells visible in both sections are numbered in both photographs in order to illustrate the method of identifying the same cells in neighbouring sections From Table 11 it can be seen that there is significant positive correlation between the intensities of AChE and E-s ns E activities in individual cells the correlation coefficient being 0 22.

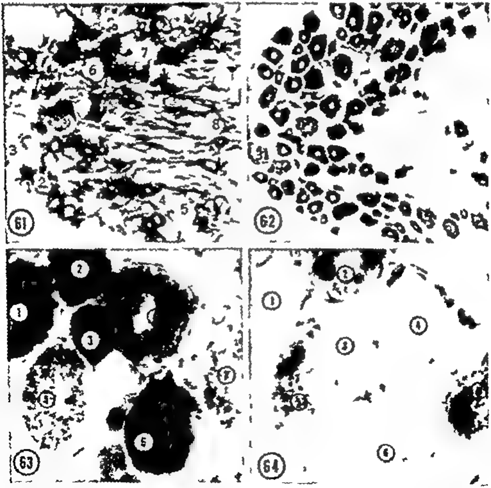


Fig 61 Cholinesterases in a fresh section. Acetylthiocholine no inhibitor × 120
Fig 62 E-s ns F in the neighbouring section α-Naphthyl acetate eserine 10^{-5} M × 120
Some of the same cells visible in both sections are indicated with numbers
Fig 63 AChE in a fixed section Acetylthiocholine iso-OMPA 10^{-7} M × 480
Fig 64 E-r ns E in the neighbouring section α-Naphthyl acetate E 600 10^{-5} M × 480
The same cells visible in both sections are indicated with numbers

Table 12 shows the results obtained from comparison between the intensities of acetylcholinesterase and E 600-resistant non specific esterase activity. Fig 63 shows a section in which AChE is demonstrated and Fig 64 shows the neighbouring section demonstrating E r ns E in the same cells. There is no significant correlation between the activities of AChE and E r ns E.

TABLE 11 *Correlation of visually estimated intensities of E 600 sensitive non specific esterase activity (y) and acetylcholinesterase activity (x) in fresh sections. The number of cells falling into each cathegory is given*

E s ns E activity (arbitrary units) \ AChE activity (arbitrary units)	Negative (0)	Weak (1)	Moderate (2)	Strong (3)	Sum
Strong (3)	2	5	19	10	36
Moderate (2)	15	52	72	33	172
Weak (1)	11	36	28	8	83
Negative (0)	0	[illegible]	0	0	0
Sum	28	93	119	51	291

Mean E s ns E activity = 1 84
Mean AChE activity = 1 66
Regression equation $y = 0\,16x + 1\,58$
Correlation coefficient $r = 0\,22$
Residual standard deviation = 0 61
Significance of correlation $P < 0\,001$

TABLE 12. *Correlation of visually estimated intensities of E 600 resistant non specific esterase activity (y) and acetylcholinesterase activity (x) in fixed sections. The number of cells falling into each cathegory is given*

E r ns E activity (arbitrary units) \ AChE activity (arbitrary units)	Negative (0)	Weak (1)	Moderate (2)	Strong (3)	Sum
Strong (3)	9	12	13	4	38
Moderate (2)	3	11	12	[illegible]	34
Weak (1)	6	7	5	6	24
Negative (0)	1	2	5	0	8
Sum	19	32	35	18	104

Mean E r ns E activity = 1 98
Mean AChE activity = 1 44
Regression equation $y = 0\,05x + 1\,91$
Correlation coefficient $r = 0\,06$
Residual standard deviation = 0 96
Significance of correlation $P > 0\,1$

Cytophotometric Estimations

In the above described correlation studies the best correlations observed were between the cell diameter and the AChE or AcPh activity It was therefore of obvious interest to study also the eventual correlation between AChE and AcPh activities Such a study was planned carefully using not only the subjective visual method of estimating the enzyme activity but also cytophotometric density measurements to record the darkening due to the enzyme reaction Blind triplicate estimations made with visual and photometric methods on the same cells furnished valuable information of the value of the employed methodology

Considerable variation was observed in the results obtained in different days or indeed in one and the same day between different sets of sections Such variation is probably mainly due to differences in the thickness of the frozen sections employed Therefore the results were separately analyzed for each pair of sections

The primary data obtained from one such pair of sections is given as an example in Tables 13 and 14 Of this data correlations calculated for AChE AcPh and cell diameter are illustrated in Figs 65 66 and 67 A typical pair of neighbouring sections thus analyzed is shown in Figs 68 and 69 Seven such sets of data were collected the total material thus comprising 269 cells whose intensities of AChE and AcPh activities were estimated in neighbouring sections

Subsequent tables have been obtained by calculation from the primary material of all the seven sets of cells Tables 15 16 and 17 giving information relevant from the methodological point of view Tables 18 19 20 and 21 showing correlations between the activities of AChE and AcPh and the cell diameter

It is evident from Table 15 that reproducibility of both the visual method and the photometric method is remarkably high. Tables 16 and 17 indicate a high correlation between these two methods

Since the photometric method is objective only the results obtained with it were used for the study of correlations between enzyme activities and the cell diameter Correlation data of AChE activity and cell diameter is presented in Table 18 Although the mean density of the AChE »staining» varied as much as from 0 32 to 0 79 in the different sets of measurements remarkably high negative correlations were observed in each independent set the value of the correlation coefficients r ranging from —0 46 to —0 69

Table 19 presents in an analogous way the relationships between the AcPh activity and the cell diameter Again significant correlations are observed in all sets the values of r varying between —0 49 and —0 69

Table 20 shows the regression of AcPh activity on AChE activity and Table 21 presents the same data in reverse *i e* the regression of AChE activity on AcPh activity Correlation coefficients for each set are of course the same in both tables showing significant positive correlations between the activities of these two enzymes The correlation coefficient varied from 0 44 to 0 71 being generally about 0 5 in spite of marked variations in the mean optical density of AChE »stained» cells

TABLE 13 *Primary data of section set number 7 Of each ganglion cell the measured diameter and triplicate visual and photometric values of the intensity of acetylcholinesterase activity are given Visual estimates are arbitrary units photometric values are given in terms of optical density Real diameters in microns can be obtained by multiplying with 5*

Cell number	Diameter	Visual acetylcholinesterase activity			Photometric acetyl cholinesterase activity		
		I	II	III	I	II	III
1	11	1	1	1	0.23	0.33	0.30
2	6	2	2	2	0.57	0.59	0.58
3	8	2	2	2	0.60	0.64	0.64
4	6	3	3	3	0.71	0.81	0.79
5	8	2	2	2	0.68	0.68	0.66
6	9	1	1	1	0.27	0.30	0.29
7	4	2	2	2	0.63	0.75	0.65
8	6	2	2	2	0.54	0.57	0.67
9	6	3	3	3	1.07	1.09	1.02
10	7	2	2	2	0.68	0.66	0.65
11	7	2	2	2	0.60	0.53	0.58
12	7	3	3	3	0.87	0.92	0.88
13	4	2	2	2	0.58	0.54	0.64
14	4	3	3	3	0.93	0.94	0.87
15	5	3	3	3	0.88	0.93	0.93
16	7	2	2	2	0.66	0.68	0.64
17	5	3	3	3	0.93	1.05	1.03
18	3	2	2	2	0.69	0.92	0.96
19	6	3	3	3	1.05	1.00	0.99
20	8	1	1	1	0.58	0.57	0.49
21	6	3	3	3	0.90	0.84	0.87
22	4	3	3	3	0.90	0.91	0.77
23	4	3	3	3	0.87	0.85	0.85
24	9	2	1	2	0.50	0.43	0.42
25	9	1	1	1	0.31	0.32	0.30
26	8	2	2	2	0.77	0.74	0.77
27	6	3	3	3	1.11	1.10	1.00
28	5	3	3	3	1.12	1.11	1.16
29	7	2	1	2	0.64	0.58	0.66
30	8	2	2	2	0.65	0.57	0.59
31	9	2	2	2	0.63	0.65	0.61
32	6	2	2	[illegible]	0.78	0.78	0.76
33	9	2	2	2	0.69	0.72	0.73
34	3	3	3	3	1.05	1.05	1.19
35	4	3	3	3	1.15	1.15	1.12
36	3	2	2	3	1.20	0.96	0.92
37	8	2	2	2	0.87	0.80	0.80
38	[illegible]	3	3	3	1.19	1.25	1.16
39	4	3	3	3	1.09	1.23	1.12
40	6	3	3	3	1.26	1.27	1.30
41	7	2	2	2	0.91	0.89	0.92

TABLE 14 *Primary data of section set number 7 This data is collected from the neighbouring section to the section presented in Table 13, the same cells being involved Of each ganglion cell the measured diameter and triplicate visual and photometric values of the intensity of acid phosphatase activity are given Visual estimates are arbitrary units photometric values are given in terms of optical density Real diameters in microns can be obtained by multiplying with 5*

Cell number	Diameter	Visual acid phosphatase activity			Photometric acid phosphatase activity		
		I	II	III	I	II	III
1	11	2	2	2	0 65	0 59	0 56
2	9	2	2	2	0 48	0 52	0 56
3	9	1	1	1	0 37	0 38	0 41
4	6	2	2	2	0 68	0 65	0 70
5	7	1	1	1	0 37	0.39	0 41
6	7	2	2	2	0 51	0 49	0 47
7	6	1	1	1	0 28	0 23	0 24
8	6	1	1	1	0 34	0 37	0 35
9	5	2	2	2	0 55	0 69	0 60
10	9	1	1	1	0 49	0 47	0 43
11	8	1	1	1	0 65	0 54	0 55
12	6	2	2	2	0 90	0 90	0 90
13	5	3	3	3	1 08	1 05	1 03
14	5	3	3	3	1 12	1 07	1 12
15	4	3	3	3	1 00	0 99	1 02
16	5	1	1	1	0 50	0 44	0 42
17	3	2	2	1	0 58	0 52	0 49
18	4	2	2	2	0 49	0 51	0 41
19	4	2	2	2	0 67	0 67	0 62
20	6	1	1	1	0 64	0 61	0 57
21	6	2	1	1	0 58	0 50	0 56
22	3	3	3	3	0 58	0 70	0 70
23	3	2	1	1	0 47	0 43	0 42
24	8	2	2	2	0 47	0 39	0 43
25	9	1	1	1	0 38	0 37	0.32
26	8	2	2	2	0 59	0 55	0 54
27	7	2	2	2	0 67	0 62	0 62
28	3	2	2	2	0 57	0 61	0 63
29	9	1	1	1	0 34	0 35	0 35
30	7	1	1	1	0 46	0 43	0 43
31	7	1	1	1	0 45	0 38	0 39
32	7	2	2	2	0 75	0 61	0 60
33	9	2	2	[illegible]	0 74	0 59	0 48
34	6	3	3	3	1 09	0 97	1 00
35	5	3	3	3	1 15	1 02	0 93
36	3	3	3	3	1 06	0 97	1 00
37	7	1	1	1	0 40	0 41	0.37
38	4	2	2	2	0 75	0 70	0 65
39	5	3	3	3	1 09	0 98	1 03
40	7	2	2	2	0 74	0 68	0 58
41	9	2	2	[illegible]	0 50	0 46	0 47

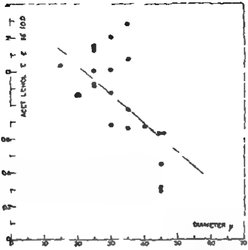


Fig 65 Correlation between the photometrically measured optical density of acetylcholinesterase activity and the cell diameter with the corresponding regression line This diagram is based on the data obtained from section set seven

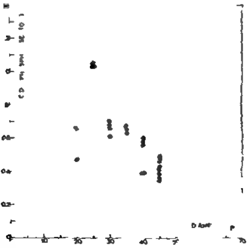


Fig 66 Correlation between the photometrically measur densi acti ity and the cell diameter with the corresp ressic is based on the data obtained from section set

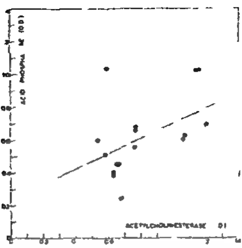


Fig 67 Correlation between the optical density of acid phosphatase activity and the optical density of acetylcholinesterase activity with the corresponding regression line This diagram is based on the data obtained from section set seven.

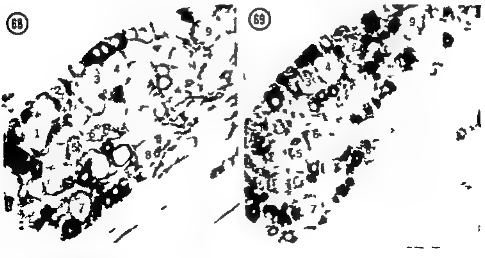


Fig 68 Cholinesterases in a fixed section Acetylthiocholine no inhibitor × 125
Fig 69 Acid phosphatase in the neighbouring section × 125
Some of the same cells visible in both sections are indicated with numbers

TABLE 15 *Reproducibility of the visual and photometric methods for estimating the intensity of the enzyme activity*

Variable measured	Number of triplicates	Standard deviation within triplicates $s(x)$	Mean total $\bar{x}$	Coefficient of variation (%) $100\frac{s(x)}{\bar{x}}$
Visually measured intensity of AChE activity	269	0 28	1 98	14
Photometrically measured intensity of AChE activity	269	0 04	0 49	8
Visually measured intensity of AcPh activity	269	0 21	2 00	10
Photometrically measured intensity of AcPh activity	269	0 05	0 66	8

TABLE 16 *Correlations between visual and photometric estimations of the intensity of acetylcholinesterase activity in different sets of sections*

Section set	Number of cells	Correlation coefficient	Significance of correlation
1	25	0 93	$P<0\,001$
2	32	0 94	$P<0\,001$
3	44	0 94	$P<0\,001$
4	59	0 92	$P<0\,001$
5	38	0 94	$P<0\,001$
6	30	0 93	$P<0\,001$
7	41	0 94	$P<0\,001$

TABLE 17 *Correlations between visual and photometric estimations of the intensity of acid phosphatase activity in different sets of sections*

Section set	Number of cells	Correlation coefficient	Significance of correlation
1	25	0 84	$P<0\,001$
2	32	0 88	$P<0\,001$
3	44	0 89	$P<0\,001$
4	59	0 89	$P<0\,001$
5	38	0 87	$P<0\,001$
6	30	0 86	$P<0\,001$
7	41	0 88	$P<0\,001$

TABLE 18 *Regression of photometrically measured optical density of acetylcholinesterase activity (y) on the cell diameter (x) Real diameters in microns can be obtained by multiplying with 5*

Section set	Number of cells	Mean AChE activity (optical density)	Mean diameter (mm)	Regression equation $y = bx + a$	Correlation coefficient r	Residual standard deviation	Significance of correlation
1	25	0.36	6.32	$y = -0.027x + 0.53$	-0.52	0.10	$P < 0.01$
2	32	0.39	6.50	$y = -0.052x + 0.73$	-0.69	0.12	$P < 0.001$
3	44	0.35	6.32	$y = -0.037x + 0.58$	-0.58	0.12	$P < 0.001$
4	59	0.32	6.73	$y = -0.025x + 0.48$	-0.47	0.11	$P < 0.001$
5	38	0.69	6.13	$y = -0.054x + 1.02$	-0.46	0.22	$P < 0.01$
6	30	0.58	7.10	$y = -0.048x + 0.92$	-0.68	0.14	$P < 0.001$
7	41	0.79	6.88	$y = -0.086x + 1.38$	-0.65	0.19	$P < 0.001$

TABLE 19 *Regression of photometrically measured optical density of acid phosphatase activity (y) on the cell diameter (x) Real diameters in microns can be obtained by multiplying with 5*

Section set	Number of cells	Mean AcPh activity (optical density)	Mean diameter (mm)	Regression equation $y = bx + a$	Correlation coefficient r	Residual standard deviation	Significance of correlation
1	25	0.73	6.32	$y = -0.050x + 1.05$	-0.49	0.22	$P < 0.01$
2	32	0.71	6.50	$y = -0.076x + 1.21$	-0.69	0.17	$P < 0.001$
3	44	0.69	6.32	$y = -0.069x + 1.12$	-0.68	0.17	$P < 0.001$
4	59	0.75	6.73	$y = -0.043x + 1.04$	-0.49	0.18	$P < 0.001$
5	38	0.52	6.13	$y = -0.052x + 0.83$	-0.55	0.17	$P < 0.001$
6	30	0.58	7.10	$y = -0.046x + 0.91$	-0.58	0.18	$P < 0.001$
7	41	0.61	6.88	$y = -0.063x + 1.05$	-0.52	0.20	$P < 0.001$

ber ... lls	Mean AcPh activity (optical density)	Mean AChE activity (optical density)	Regression equation $y = bx + a$	Correlation coefficient r	Residual standard deviation	Significance of correlation
	0 73	0 36	$y = 1\,06x + 0\,35$	0 52	0 21	$P<0\,01$
	0 71	0 39	$y = 0\,73x + 0\,43$	0 51	0 20	$P<0\,01$
	0 69	0 35	$y = 0\,69x + 0\,45$	0 44	0 21	$P<0\,01$
	0 75	0 32	$y = 0\,74x + 0\,52$	0 45	0 18	$P<0\,001$
	0 52	0 69	$y = 0\,37x + 0\,26$	0 45	0 18	$P<0\,01$
	0 58	0 58	$y = 0\,82x + 0\,10$	0 71	0 15	$P<0\,001$
	0 61	0 79	$y = 0\,46x + 0\,25$	0 50	0 20	$P<0\,001$

Regression of photometrically measured optical density of acetylcholinesterase ...tivity (y) on optical density of acid phosphatase activity (x)

...ber ...ells	Mean AChE activity (optical density)	Mean AcPh activity (optical density)	Regression equation $y = bx + a$	Correlation coefficient r	Residual standard deviation	Significance of correlation
...5	0 36	0 73	$y = 0\,26x + 0\,17$	0 52	0 10	$P<0\,01$
...2	0 39	0 71	$y = 0\,35x + 0\,14$	0 51	0 14	$P<0\,01$
...[illegible]	0 35	0 69	$y = 0\,28x + 0\,15$	0 44	0 13	$P<0\,01$
...[illegible]	0 32	0 75	$y = 0\,27x + 0\,12$	0 45	0 11	$P<0\,001$
...3	0 69	0 52	$y = 0\,55x + 0\,41$	0 45	0 22	$P<0\,01$
...[illegible]	0 58	0 58	$y = 0\,61x + 0\,22$	0 71	0 13	$P<0\,001$
...[illegible]	0 79	0 61	$y = 0\,55x + 0\,45$	0 50	0 22	$P<0\,001$

DISCUSSION

VALIDITY OF THE OBSERVATIONS

Since the measurement of the cell diameter was based only on the part of the cell visible in a given section some of the diameters obtained are likely to be too small This inaccuracy in diameter measurement can as such be expected to cause a numerically too low coefficient

However since tangential fragments of large cells may sometimes in the present study have been taken as small cells a false negative correlation between the enzyme activity and the cell size could be expected if the reaction had been concentrated mainly in the periphery of the cell Concerning acetylcholinesterase which was observed [redacted] bear a negative correlation to the cell size such an error can be excluded because the enzyme activity was evenly distributed throughout th[redacted] cell

Acid phosphatase on the other hand tended to appear in strongly positive peripheral aggregations in the large cells and thus may have somewhat contributed to the negative correlation observed between the enzyme activity and the cell size tangential parts of large cells with such granules being taken as small strongly positive cells However caps of large cells included in the small cell population were certainly few because only such cells were selected which were present in two neighbouring sections Moreover most of the small cells measured had their nuclei in the section and their cytoplasm showed an evenly distributed intense AcPh reaction

While E r ns E activity also was often seen in peripheral aggregations no significant correlation between this type of enzyme activity and the cell size was seen This suggests that the discussed »cap effect» did not have practical significance as a possible cause of false negative correlation between the cell size and the enzyme activity

As a subjective method, the visual estimation of enzyme activity is *a priori* open to criticism However realizing that only limited information can be obtain ed using such method assessment of the reaction intensity as negative, weak moderate or strong is justified so long as the evaluation can be shown to be reproducible In the present study when the error of the method was calculated with the aid of blind triplicate estimations it was found to be surprisingly small Thus the method is highly reproducible. The validity of the visual estimation was also established by comparison with photometric measurements the two sets of data agreed closely

le both the visual and the photometric estimation of enzyme activities are reproducible they only measure the optical density of the cells Apart he distribution error, which is only partly allowed for, error due to non relationship between the enzyme activity and the density of the pigment histochemical reaction is also included Therefore the results of both nethods are subject to definite limitations in the quantitative sense How t is by such means possible to characterize with numerical values different of cells and thus rank them according to the reaction intensity For the e of the present study such a possibility is of definite value For further ents on this general problem see Eränkö (1955)

IZE AND ENZYME ACTIVITY OF INDIVIDUAL CELLS

ative correlations were observed between the cell diameter and the intensity h the AChE and the AcPh reaction On the other hand the AChL activity ositively correlated with both AcPh and E-s ns E activity The »cap effect» ied above cannot be responsible for such an observed correlation All of correlations are highly significant *i e* the correlations observed were not mere chance Even if the observed correlations can therefore be used as ors of a *possible* causal relationship between the correlated variables it be emphasized that a significant correlation does by no means indicate a relationship

correlation coefficients obtained ranged from 0 19 to 0 71 the best correla being observed between photometrically measured AChE activity cPh activity A correlation coefficient — 0 38 was found comparing AcPh y with the cell diameter this was the best correlation coefficient obtained isual estimations That the correlation coefficients obtained when the e activity was measured visually were as a rule lower than those from metric estimations is understandable because of the lower accuracy of the r method

present study clearly indicates that there is a significant tendency of smaller o exhibit higher AChL activities than the larger cells This was the case ily in regard to AChE but AcPh as well On the other hand the intensities RNA E-s ns E and E-r ns E reactions were independent of the cell size le earlier studies are not available in which quantitative measurements een carried out of the intensity of any histochemically demonstrable esterase ons in spinal ganglion neurones there are some previous observations point in the same direction as the present study

eral writers (Colmant 1959 Tewari and Bourne 1962a Galabov *et al* have reported that the small cells in the spinal ganglion show higher AChE cPh activities than the large ones These studies were carried out by em g qualitative visual estimation only

only previous quantitative study of which I know (Giacobini 1959a was based on microchemical determinations of AChE activity in single ganglion cells removed by microdissection and treated individually in ian divers Giacobini s data clearly indicate a negative correlation between

the cell size and the AChE activity although he mainly emphasized the presence of two separate cell populations one consisting of very active and the other weakly active or inactive cells

Why the smaller cells show higher AChE and AcPh activities is unclear Some speculations can nevertheless be presented

Different functions have been proposed for the small and the large cells The small spinal ganglion cells have been thought to be concerned mainly with the reception of pain temperature and pressure the larger ones with touch, deep sensation and proprioception (Scharf 1958 Grosby *et al* 1962 Swanson *et al* 1965) These functional differences might perhaps be associated with differences in the enzymatic composition of the cells although of course no direct interdependence between the sensory modalities and the histochemical properties of the cells can be expected

Metabolic changes due to periodic exhaustion and restitution of the cells might be a further tentative explanation for the intensity variation between individual cells Tewari and Bourne (1962a and c) have indeed introduced a hypothesis of a metabolic cycle taking place in spinal ganglion cells as indicated by differences which they observed in the cytoplasmic distribution of several enzymes and in the size of the nerve cell nucleoli The present writer (unpublished observations) was unable to confirm any of these differences which formed the main body of evidence for the hypothesis of Tewari and Bourne

Giacobini (1959a) suggested that the spinal ganglion cells which exhibited a very high total AChE activity per cell might belong to a cholinergic reflex arc, the AChE negative cells should according to him be non-cholinergic Although the cells showing the highest AChE activity in the present study may indeed be cholinergic the others having a main transmitter other than ACh the presence of cells with intermediate reaction does not fit in with this dualism

On the other hand the presence of AChE in non-cholinergic cells could be explained according to Koelle s theory (1962) that also in non cholinergic cells ACh would play an initiating role in the formation of the nerve impulse and therefore also ACh and AChE would be needed in these neurones Indeed some of the definitely adrenergic cells in rat s sympathetic ganglia contain abundantly AChE (Eranko and Harkonen 1964 Harkonen 1964) Speculating further one might assume the presence of different types of neurones with acetylcholine and another transmitter the concentration ratios varying from one individual cell to another In such a case the »less-cholinergic» cells could be expected to contain less AChE

The tendency of smaller cells to show higher AChE activity than the larger ones can be interpreted also otherwise assuming this time that ACh *is* the transmitter in the sensory nerve terminals and that AChE is needed there The amount of ACh and AChE needed in each cholinergic nerve terminal could be assumed to be the same regardless of the distance between the perikaryon and the nerve terminal On the other hand cell bodies belonging to the spinal ganglion cells which have longer dendrites have been shown to be in general larger than those having shorter processes (Haggqvist and Lindberg 1961) Since a large neurone with a long dendrite (or axon) must carry out an active protein synthesis the cell body must contain a large amount of RNA since RNA does not usually reach the cell processes Thus it would be rather locigal that a neurone having

While both the visual and the photometric estimation of enzyme activ. highly reproducible, they only measure the optical density of the cells from the distribution error which is only partly allowed for error due linear relationship between the enzyme activity and the density of the p due to histochemical reaction is also included Therefore the results these methods are subject to definite limitations in the quantitative sense ever it is by such means possible to characterize with numerical values d types of cells and thus rank them according to the reaction intensity purpose of the present study such a possibility is of definite value For comments on this general problem, see Eranko (1955)

SIZE AND ENZYME ACTIVITY OF INDIVIDUAL CELL

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long processes should have a large perikaryon If then each neurone has the same amount of AChE at its nerve endings regardless of the size of the whole neurone a cell with shorter processes and smaller perikaryon but the same total amount of AChE would have a higher cytoplasmic concentration of the enzyme and it would exhibit a more intense histochemical AChE reaction than a larger cell with long processes

The intensities of AChE and AcPh reactions showed a highly significant positive correlation with each others the correlation coefficient being fairly high when photometric measurements were used This correlation is surprising especially because the intracellular localization of these two enzymes is different (Novikoff and Essner 1962, Torack and Barrnett 1962 Novikoff 1963 Lewis and Shute 1964) It may indicate that both of them are involved in different but functionally linked metabolic processes in the cell

The small but significant positive correlation between AChE and E s ns E is not easier to explain Both of these enzymes are considered to be attached to the same cytoplasmic organelle *i e* the endoplasmic reticulum (Aldridge and Johnson 1959 Novikoff 1961 Shnitka and Seligman 1961 Torack and Barrnett 1962) In this connexion it is of interest to note that in the present electrophoretic studies these two types of activity were found together in several sites of the zymogram This might indicate that a part of the AChE and E-s ns E activities is attached to the same protein or due to the same multi locus enzyme If this were the case variable amounts of such a protein in individual cells would cause unless masked by non paired AChE or E s ns E activity a correlation between the intensities of AChE and E s ns E activity The lack of observed correlation between AChE and RNA AChE and E-r ns E as well as RNA and E r ns E respectively hardly requires even speculative comments

In conclusion it has been possible to provide only highly tentative explanations for the principal observations of the present study, *i e* that both AChE and AcPh show a significant negative correlation to the cell size and a significant positive correlation with each other However the observations themselves are interesting, definitely proving marked dissimilarities between individual spinal ganglion cells It is to be hoped that an adequate explanation for them will be disclosed in future studies if not by the present author perhaps by somebody else with a larger brain and eventually more enzyme activity in it

SUMMARY

The present study was undertaken to elucidate the distribution of different carboxylic esterases and acid phosphatase in the spinal ganglion of the rat Since hydrolytic enzymes were found to occur in cytoplasmic granules a fuller study of these granules was undertaken Extreme variation observed in the enzyme activities of individual ganglion cells further prompted a quantitative study correlating in individual cells hydrolytic enzymes with each others and with the cell size

Carboxylic esterases The carboxylic esterases were histochemically studied in fresh and fixed sections and in starch slabs after electrophoresis using acetylthiocholine iodide butyrylthiocholine iodide α naphthyl acetate α naphthyl butyrate, naphthol AS D acetate and 4-chloro-5 bromoindoxyl acetate as substrates and 284 C 51 *iso*-OMPA eserine and E 600 as selective inhibitors The esterases were classified with the aid of inhibitors into four groups 1) acetylcholinesterase (AChE) 2) non specific cholinesterase (ns ChE) 3) E 600-sensitive non specific esterase (E-s ns E) and 4) E 600 resistant non specific esterase (E-r ns E)

AChE activity was demonstrable in the cytoplasm of ganglion cells and in some nerve fibres Activity in individual ganglion cells varied from zero to intense

Ns ChE activity was mainly limited to the structures between the perikaryons of the neurones consisting of satellite and Schwann cells and capillaries and only few ganglion cells exhibited any activity

E s ns E activity was best demonstrated in fresh sections in which all the ganglion cells exhibited activity but again great variation in the intensity between individual ganglion cells was seen Satellite cells also contained this kind of activity

E r ns E was localized mainly in cytoplasmic granules of the neurones and was demonstrable after formalin fixation only it was thus concluded to be highly soluble *i e* lyo esterase Again variations in the intensity between different neurones were found

Acid phosphatase (AcPh) The distribution of AcPh clearly differed in fresh and fixed sections In fresh sections the reaction product was diffuse but in fixed sections confined to cytoplasmic granules The intensity of the reaction varied markedly from one ganglion cell to another both in fresh and in fixed sections

Cytoplasmic granules When fresh or fixed spinal ganglion sections were studied in ultraviolet light certain cytoplasmic granules were found to exhibit yellow autofluorescence Some of these granules were found to contain AcPh and/or

E-r ns E activity some others did not exhibit any such activity and there were enzymatically active granules which did not fluoresce The identity of these different types of granules with lysosomes or lipofuscin granules is discussed

Comparison of the enzyme activities and the size of individual cells The diameters of the cells were measured from photomicrographs and the intensity of the reaction in each cell was measured either visually inspecting the section under microscope or with the aid of a photometer A significant negative correlation was found between the cell size and the AChE activity ($r = -0\ 36$ with visual method $r = -0\ 46$ to $-0\ 69$ with photometric method) and between the cell size and the AcPh activity ($r = -0\ 38$ with visual method $r = -0\ 49$ to $-0\ 69$ with photometric method) No significant correlation was found between the cell size and the activities of E s ns E E r ns E or the intensity of ribonucleic acid (RNA) staining with pyronin

Comparison of the activity levels of different enzymes in individual ganglion cells Neighbouring sections were used which were treated to show different enzymes The cells visible in both sections were numbered in photographs taken from the sections and again the intensity of both reactions in each cell was measured either visually or photometrically Significant positive correlations were found between AChE and AcPh activities ($r = 0\ 44$ to $0\ 71$ measured photometrically) and between AChE and E-s ns E activities ($r = 0\ 22$ measured visually) No correlation was found between AChE and E r ns E

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POSTNATAL GROWTH OF SPINAL NERVES AND ROOTS

A morphological study in the cat with physiological correlations

By

STEN SKOGLUND AND CESAR ROMERO

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The general problem of nerve fibre development has attracted a good deal of attention since the end of the last century. Thus Ambron and Held (1895) stated that motor nerves are myelinated before sensory ones. According to several authors (see Gutner 1936 for references) the spinal nerves are myelinated from the centre towards the periphery. Another problem that has been rather extensively studied is the possible correlation of myelination and function. The pioneer work of Tilney and Casamajor (1924) on the sequence of myelination in the central nervous system was later extended by Langworthy (1929) in a series of studies on the relation of function to myelination. Another study of importance in connection with the work to be presented is that of Kingsbury (1932) in which he convincingly shows the cephalo-caudal development of the neural tube in chick, cat and dog.

The above mentioned works usually deal with the morphological aspects of the problem of postnatal development and the functional applications of the results are with few exceptions mere speculations. A series of work on the postnatal development of spinal reflex activities in the kitten (Skoglund 1960 a, b, c, d and e) have led to the conclusion, later confirmed by Wilson (1962), that the gradual appearance of different reflex mechanisms can be correlated to the maturity of the peripheral nerves and their central connections. Earlier investigations on the postnatal development of the nerve fibres in the cat are practically lacking and investigations in other species are scanty and usually deal with the sequence of myelination in different systems (Tilney and Casamajor 1924) and/or comparisons between species (Duncan 1934). Futhermore in these investigations, without any exceptions, only spinal roots have been studied. Thus a systematic study of the progressive development of peripheral nerves and spinal roots in the cat is lacking. In view of the experimental findings mentioned above such an investigation seems highly desirable to see whether any closer correlation between morphology and function can be obtained. Furthermore the pattern of morphological maturation might give information on general principles of growth and reveal where further physiological investigations are likely to give new information on developmental mechanisms. For the correct evaluation of earlier results and those obtained a short review of the literature seems pertinent.

Review of the literature

In reviewing the earlier literature regarding the postnatal development of the peripheral nerves only those works will be discussed in detail that have lead to more generalized opinions regarding the mode of the postnatal growth of nerve fibres. Many of these opinions have very often arisen out of observations made in connection with experimental works regarding other aspects of the peripheral nervous system than development. This is for instance the case with the works of Hardesty (1899, 1900) in which he studied the number and arrangements of the fibres forming the spinal nerves in the frog. He found that the number of nerve fibres in the dorsal roots increases more rapidly than in the ventral roots with increasing weight of the frog. Similar observations regarding the increase in number of ventral root fibres with increasing weight of the frog had been made earlier by Birge (1882). This author went even that far as to determine the exact increase in number of ventral root fibres per each gram of increase in weight. Furthermore he determined that the average diameter of the fibres in the roots of younger specimens was less than in the older ones and expressed the opinion that during growth small fibres thickens into larger ones. Korybutt Daskiewicz as early as 1678 studied the ingrowing of new fibres in the sciatic nerve of the frog.

With the exception of Birge's work (1882) the above mentioned studies only dealt with the increase in number of the nerve fibres with increasing age. This aspect of postnatal growth actually dominated the developmental studies after Hardesty. Thus Hatai (1902, 1903) restated that the increase in number of ventral root fibres in the cat with increasing age is more rapid between 10 and 30 days than after this age. Furthermore he stated contrary to the findings of Birge (1882) and Hardesty (1899) in the frog, that the number of fibres increases more rapidly in the ventral roots of cats than in the dorsal roots. Hatai thought that this difference is one of species, between frog and cat. Such a difference might exist, although it is not proven by these works. Actually one is apt to think that the reason for the different findings might be the large differences between different individuals. That is not at all accounted for by the small number of animals used in these works. A really striking example of this kind is the work of Schiller (1889) in which he compared the number of fibres in the occulomotor nerve of *three* newborn kittens and *one* adult cat and con-

cluded that the increase in number during the development was one of 3 %

The lack of statistical treatment of, and even statistical viewpoints on the material which is not anything particular for the works on the nervous system of that time (see Schwieler and Skoglund 1964) lead Boughton (1906) to ask whether the small increase in number of fibres in the occulomotor nerve as found by Schiller (1889) in the cat compared with other authors reports of a more than 50 % increase in number of fibres in spinal roots depends upon a difference between cerebral and spinal nerves Alternatively this might according to Boughton also be a difference between the cat on the one hand and the frog and the rat on the other When Boughton sets out to investigate these particular questions he comes to the conclusion that there is a 75 % increase of fibres in the occulomotor nerve of the rat from 11 grams to 414 grams bodyweight Looking into his data it is seen that in the 13 animals that he used there is a 71 % increase at 77 grams 83 % at 113 grams and 92 % in another animal at 113 grams This only confirms what has been said above about individual variations and stresses how worthless such studies are when very great materials are not used and handled with statistical methods Boughton s conclusions regarding the increase in number of nerve fibres in the occulomotor nerve of the cat are based on an even smaller number of animals than was used in the work on the rat and for the reasons given above cannot be accepted as evidence In the same work Boughton also considered the increase in size of the nerve fibres and concluded that there is a continuous increase of the size throughout life He furthermore states that the small nerve fibres never attain the size of the large fibres This he thinks depends upon that they start their development after the period of most rapid growth Boughton s differentiation of the fibres in small and large is quite unintelligible and was puzzling even to his contemporaries (see Dunn 1912) why his statement that the large and the small fibres should increase in diameter at the same rate escapes our judgement

In 1912 Dunn published an extensive paper dealing with the influence of age sex and weight on the number of medullated nerve fibres and on the size of the largest fibres in the ventral root of the second cervical nerve of the albino rat She shows that the number of fibres increases up to 30 days postnatally After that time there is no uniform increase but a great variation of the number of fibres in different animals This supports the opinion given above that the individual variations in mature ages are too great to allow any conclusion about the development of the number of fibres unless a very great material is used Dunn also concludes that there

is a better relation between age and number of fibres than between the latter and body weight in contrast to Boughton (1906) Looking into Boughton s own figures actually shows that Dunn is right The postnatal development of the nerve fibre sizes however is less extensively studied in Dunn s paper since she has restricted the investigation to the biggest fibres She states that whereas the chief increase in number of nerve fibres occurs in the period up to puberty (70 days) there seems not to be the same relation with regard to the increase in size of the fibre Looking into her own data however reveals that there is an increase of about 8 microns up to 2 months but the consecutive increase of 6 microns takes 7 months Dunn s conclusion was evidently not right the growth of nerve fibre sizes is actually most rapid during the first period after birth

Ide in a series of paper (for references see Ide 1931) investigated the cross sectional area of different nerves and roots and correlated this to age sex and body weight He comes to the general conclusion that there are actually no sex differences with regard to growth in cats His work however has very little bearing on the problem of postnatal development since the results are based on cross sectional area of whole nerves and not on single fibre analysis

In 1934 Duncan published an extensive study on the relation of axon diameter and myelination and arrived to the conclusion that there is a critical nerve fibre size above which all fibres are myelinated Duncan also arrived to the conclusion that while the myelinated nerve fibres increase in size during development their number is continually added to by myelination of previously unmyelinated fibres a conclusion actually reached by Boughton as early as 1906 Thus Duncan s otherwise extensive work adds very little to the knowledge of the postnatal development of the spinal nerves

In 1945 Rexed published his important and extensive work on the postnatal development of the peripheral nervous system in man This work also contains a thorough investigation of the technique used for measuring nerve fibre sizes Rexed s main findings are in confirmation of earlier authors that the number and size of medullated nerve fibres increase with increasing age and that this process is not ended untill after a considerable lapse of time relatively to the length of life He also states that the rate of growth is fastest in early life Some of the details in Rexed s work are discussed in another publication by Nystrom and Skoglund (1965)

In 1948 Vizoso and Young published an investigation of the internode length and fibre diameter in developing and regenerating nerves in the rabbit Most of their speculations on the formation of myelin have nowa

days been elucidated by electronmicroscopy, but one of their main results namely a linear relation between fibre size and internodal length, seems still to be valid However the great variations in the internodal length in one and the same fibre as shown by these authors might be an artefact due to the treatment of the fibres in the dissection In the instances where Vizoso and Young find an internodal length of twice the size of all the others in the same fibre one is apt to think a node has been overlooked or deteriorated by dissection

Material and Methods

The material used was obtained from cats ranging in age from newly born to adult. Only two adult cats have been included from our large adult material for comparison, one young grown (about one year old) and one old grown (more than 5 years old). A great deal of the kittens were used for physiological experiments, why in many instances the morphological findings could be directly correlated to physiological determinations of conduction velocity. These correlations will, however, be taken up in a later publication.

The material consists mainly of nerves and roots belonging to the lumbosacral region of the spinal cord, but for comparisons some material from the cervical region is included too. Most of the material on the cervical roots, however, has been discarded, since it is rather inconsistent due to the great difficulties to obtain good cross sections from these short roots. Such technical difficulties have also been encountered when dealing with the more cranial roots in the lumbar region, why a complete material could not be obtained from this region of the cord either.

The material thus used is the spinal roots from L4 through S1, the nerves to the gastrocnemius muscle (2 branches), the nerve to the adductor longus muscle, the hamstring nerve and the quadriceps nerve except its rectus portion. Futhermore the sural nerve taken at the height of the popliteal fossa and the saphenous nerve at two sites, when it leaves the femoral nerve and at the height of the knee after it has given off its branches to the knee joint. The radial and the phrenic nerves from the cervical region have been included in the material presented here. Great care was taken to select the specimens from comparable places in different cats. The muscle nerves were taken as far as possible from the muscle, at least 1 cm from it in the small animal. This is a point of great importance because of the branching of the fibres as they approach the muscle (Eccles and Sherrington 1930).

The specimens are identified with running numbers for all animals used in the work on postnatal development and with capital letters to denote different littermates. All specimens were taken out from the living animal under Nembutal anesthesia, fixed in 0.5% osmic acid for 24 hours and dehydrated in alcohols and embedded in paraffin. Sections were cut at 3—5 microns thickness. Selected sections were then photographed on roll

film directly in the microscope and the films were then enlarged to an overall magnification of 1 000 times (Romero and Skoglund 1965). The nerve fibres were measured with a particle size analyzing machine (Zeiss TGZ 3) according to the method of Romero and Skoglund (1965). The complete material is presented in Table I—XX where the actual amount of fibres in the different groups are given. To ease reading some of the material is presented in diagrammatic form in which the fibres in the different groups have been plotted in percentages of the number of fibres measured against a scale of even microns.

The quality of the material in an investigation like this is very critical since small differences are compared. We do not want to overemphasize the technical difficulties but compared with the ease with which good cross sections can be produced for instance from nerves in newborn man (Nystrom and Skoglund, 1965) they are considerable. One reason for this is that the fascicles in the roots float apart and when just putting them together to get a cross section of the whole root all kinds of angles must be tried and still sections perpendicular to the length of all fibres are difficult to obtain. It seems as if the fibres do not even have to fall apart but undergo slight changes in their relative positions which causes all kinds of odd configurations in the cross sections. In the peripheral nerves another source of troubles is encountered which is also met with in peripheral nerves of newborn man. Here the great excess of unmyelinated fibres compared with myelinated ones seems to distort the cross sections. From this follows that a great amount of the material had to be discarded, but still measurements from some 150 000 fibres are presented.

For slight irregularities within otherwise perfect cross sections certain rules for the measurements were adopted (see Romero and Skoglund 1965). If the fibres were pearshaped the diameter was measured on its base. If the fibre was oval shaped the minimum diameter was measured (see Rexed 1944).

In the big roots many photographs were taken and amongst those a choice was made quite randomly. The peripheral nerves were, if not too large, photographed in their full extent. The number of fibres measured in each nerve depends upon the number of fibres in the specimen and the distribution of the fibre diameters. These points of great importance for the statistical treatment of the material will be extensively treated in a publication to appear (Romero, Skoglund and Taube). No fixed number of fibres to be counted was determined from the beginning of the work not to squeeze the material beyond a point where it was no longer acceptable for technical reasons like bad staining or imperfectness in crosssectioning.

Results

General development

When studying the cross sections of nerves or roots in the newborn cat one very conspicuous thing compared with the adult nerve besides the difference in fibre size is the large yellow stained areas devoid of well visible myelinated fibres (cf Rexed 1944 Nyström and Skoglund 1965) These areas are studded with unmyelinated and small myelinated fibres less than 1 micron Fig 1 from the sural nerve (A) and the dorsal (B) and ventral (C) roots S1 at different ages nicely illustrates the above mentioned development Furthermore some countings of myelinated fibres in the peripheral nerves at different ages have clearly shown that there is a postnatal increase of the number of myelinated fibres (cf Agduhr 1919—1920 Kjellgren 1944 and Rexed 1944)

The general development of the peripheral nerves and the spinal roots is also clearly shown in Fig 1 In the 1 day old kitten the sural nerve (A) and the dorsal (B) and the ventral (C) roots have fibres of about the same size (cf Skoglund 1960 b) In the sural nerve and the dorsal root the areas devoid of nerve fibres seem larger At a later stage of development—23 days—the fibres in the roots are bigger than in the sural nerve and there are practically no fibre free areas in the ventral root At 45 days the fibres in the roots are much bigger than in the sural nerve and no yellow areas can be seen any longer In the sural nerve fibres of all sizes seem to be present In the dorsal root the same holds true but here one sees to the right in the picture bundles of many small fibres In the ventral root on the other hand there are big fibres and small fibres but very few in between Thus at this stage—45 days—the sural nerve and the roots have reached their adult pattern and it is only an increase in size of the nerve fibres that takes place after this until they have reached their final adult values

Spinal roots

In the following the general development of the spinal roots will be considered The different roots have been tabulated separately so that the increase in size of the fibres in each segment can be easily seen in Table

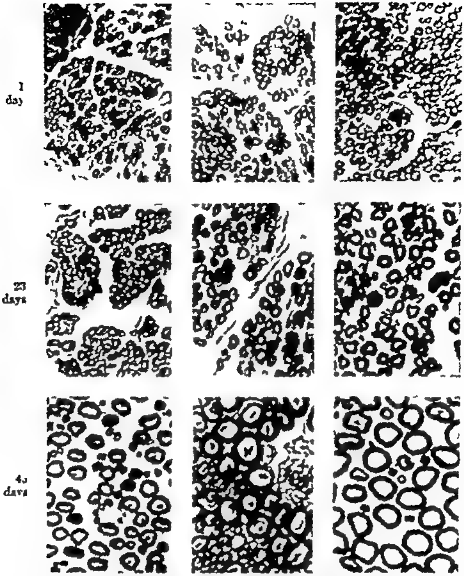


Fig 1 Comparison of cross-sections from the sural nerve (A) the dorsal root (B) and the ventral root (C) S1 at three different ages given in the figure Magnification 1,000 times For further explanation see text.

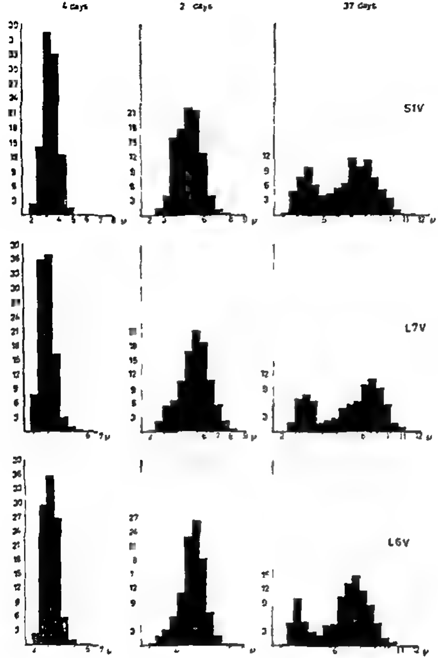
I—X (p 34) Furthermore some of the roots have been selected and presented in histograms for illustration

Development of the ventral roots Looking in Table II IV VI VIII and X for the ventral roots we can see that the trend in the development is about the same in the different roots To illustrate this the ventral roots

S1, L7 and L6 are presented as histograms in Fig 2—4 at the ages given in the figure. As is seen in the 4-days old animal there is a peak around 3—4 microns which is slowly shifted to reach 5 microns at 26 days. At this stage a great many of the fibres have reached above 3 microns. Then up to 37 days something new happens. From this stage two peaks can be recognized just as in the adult ventral root. One peak is lying at 2—3 microns and the other at 7—8 microns. At 52 days the peak for the small fibres is shifted to 3—4 microns and the peak for the big fibres to 8—9 microns. Then at 91 and 153 days the peak for the small fibres is at 4 microns and that for the big fibres is above 11 at 91 days and above 12 microns at 153 days. In the adult animals the peak for the smaller fibres has reached above 5 microns and that for the large fibres above 15 microns. This seems to be in good accordance with the values for the motor fibres as given by Rexed and Therman (1948) if some correction for shrinkage is made in their material because they used the Alzheimer Mann Häggquist method. It should be noted that in the young grown cat the biggest fibres do only reach 20 microns wheras in the old cat they are even above 22 microns.

Development of the dorsal roots. Considering now the dorsal root in the same fashion as the ventral ones we find quite a different picture. The whole material is presented in Table I, III, V, VII and IX and some of it in histograms in Fig 5—7. As seen in Fig 5 most of the fibres seem evenly distributed between 2—4 microns in the 4 days old animal. Then at 26 days it is seen that the fibres are spread out between 3—7 microns without any clearly indicated peaks. At this age a great many of the fibres have reached above 3 microns. At 37 days the spreading of the fibres is even more pronounced from 2—11 microns. At 52 days (Fig 6) the biggest fibres have reached 12 microns. Then at 91 days the biggest fibres have reached 16 microns but now there is a clear peak around 3 microns. This peak is even more pronounced at 153 days when the biggest fibres have reached 17 microns. Thus at this stage the histograms of the dorsal roots seem to have reached the same pattern as in the adult animal when compared with Fig 7.

Comparison of the development of ventral and dorsal roots. From the above it is clear that the development of the dorsal roots differs from that of the ventral ones in several respects (for illustration see also Fig 10—13). Firstly the fibres in the dorsal roots are more evenly distributed over the whole spectrum as pointed out earlier and illustrated in Fig 1. Secondly most of the big fibres reach above 3 microns somewhat later in the dorsal



… 2 H…gram of the …entral root S1 L7 and L6 at the different ages given in the …ure

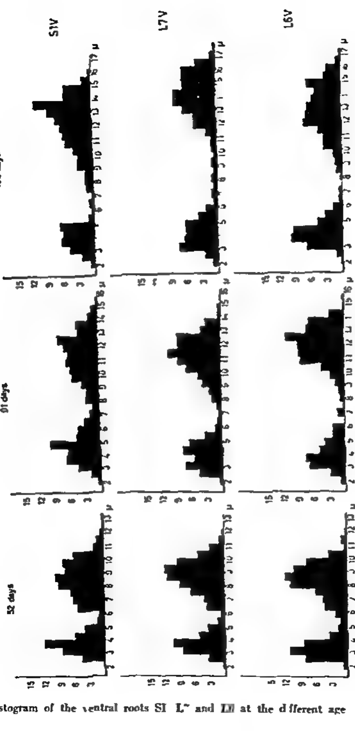


Fig 3 Histogram of the ventral roots SI L7 and L6 at the different age given in the figure

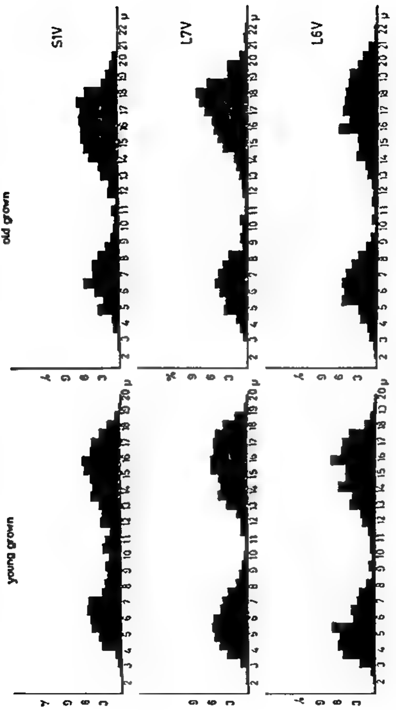


Fig 4 Histograms of the ventral roots SI L7 and L6 at two different ages given in the figure

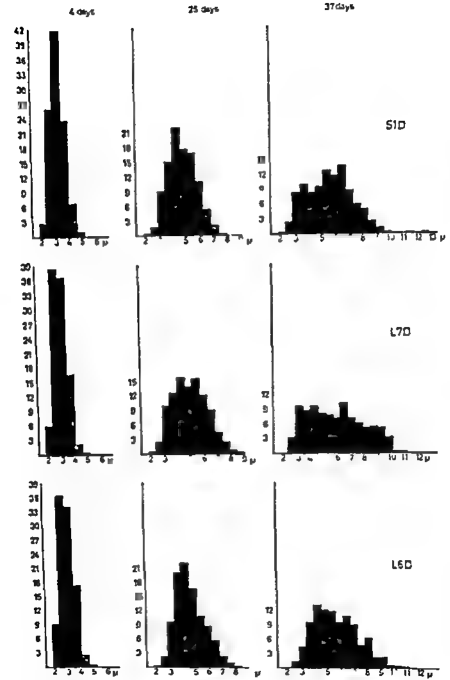


Fig 5 Histograms of the dorsal roots SI L6 and L7 at the different ages given in the figure

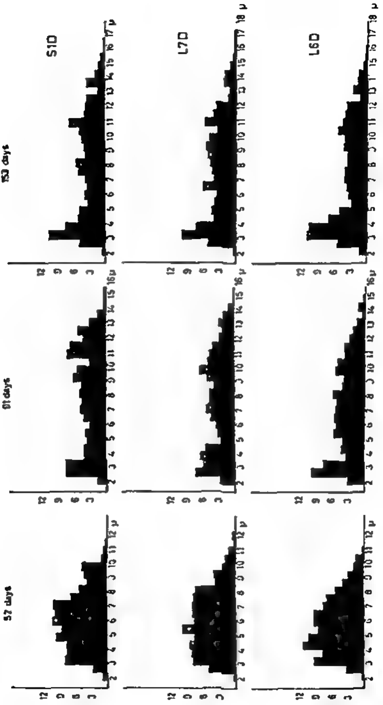


Fig 6 Histograms of the dor al roots SI L6 and L7 at the different ages given in the figure

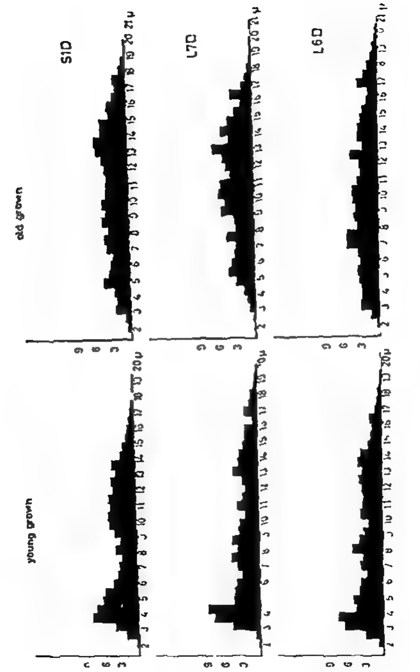


Fig 7 Histograms of the dorsal roots S1 L7 and L6 at two different ages given in the figure

than in the ventral roots (see Table I and II). This fact in combination with the observation mentioned earlier that the areas of unmyelinated fibres are less conspicuous in the ventral than in the dorsal roots add to the conclusion that the latter lag somewhat behind the former in development. This is of course only a descriptive way of characterizing the development since, as we can see, the ventral roots have bigger fibres in the adult stage. The real difference in development is shown in Fig. 8 where the peak for the biggest fibres at different stages of development are plotted against postnatal age. It is clearly seen that the ventral and dorsal roots starts from about the same fibre size but that the ventral root grows faster than the dorsal one. Furthermore it can be seen that there is a rather fast growth of the diameters in the beginning, then the development seems to run at a slower rate. This observation confirms the finding of Ide (1930). Furthermore our findings confirm the observation of Duncan (1934) that all fibres show an increase in size during growth and the reason why the peak of the small fibres seems to move less rapidly to the right in the spectrum is of course that new fibres are continually being added to left end of the spectrum as they become myelinated.

Comparison between the development in different segments. Until now we have only compared the ventral and dorsal roots with each other. If we now focus our attention on the roots of different segments, comparing dorsal with dorsal and ventral with ventral roots it is quite obvious that the material does not allow any certain conclusion about possible differences between upper and lower lumbar segments. It appears though as if the L7 roots were somewhat ahead in the development.

Peripheral nerves

General development of peripheral nerves. The peripheral nerves like the roots have been tabulated separately (Table XI—XX, p. 40) to show the postnatal growth in each nerve. Here we can easily see that the peripheral nerves follow the same pattern of growth as the roots. This is also illustrated in Fig. 9 where the peak of the biggest fibres is plotted against postnatal time in days for the medial gastrocnemius and the sural nerves. As is seen the rate of growth of the skin nerve is somewhat slower than that of the muscle nerve. This finding confirms earlier results obtained by determining the conduction rate in muscle and skin nerves at different ages (Skoglund 1960 b).

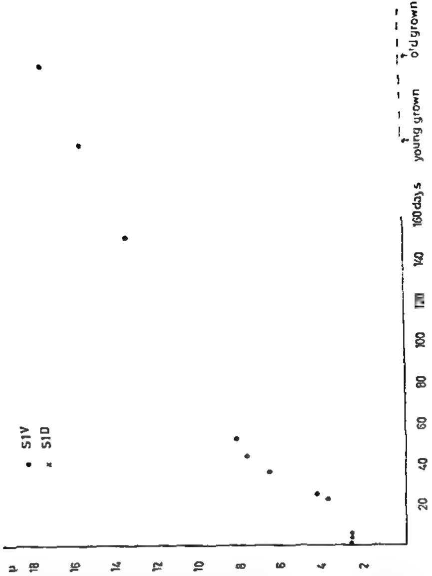


Fig 8 Graph showing the increase in size of the nerve fibres comprising the peak of the biggest fibres in the ventral root SI (filled circles) and the dorsal root SI (crosses) at different ages Note the faster growth of the ventral root fibres

Comparison between peripheral nerves and spinal roots and between different peripheral nerves Comparing the muscle nerves with the roots in the 1 day old kitten (Fig 10) shows a slight preponderance of bigger fibres in the roots If we compare the different muscle nerves at the same stage

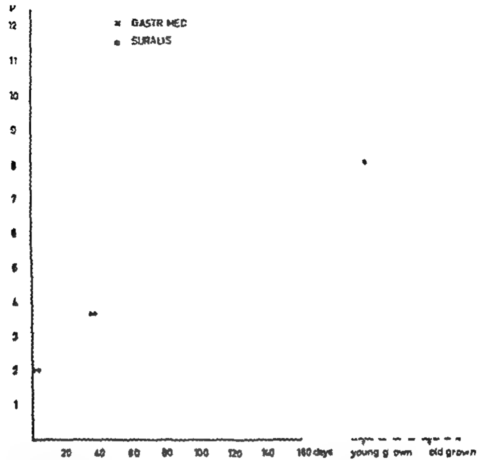


Fig. 9. Graph to show the increase in size of the fibres comprising the peak of the biggest fibres in the medial gastrocnemius nerve (crosses) and the sural nerve (filled circles) with increasing age.

there is the same tendency for a predominance of bigger fibres in the proximal nerves like the adductor compared with the nerves to the gastrocnemius muscle. The radial nerve—from the cervical region of the cord—has as big fibres as the proximal nerves. At a later stage however—20 days—the roots seem more advanced than both the proximal and distal muscle nerves and the radial nerve. Now on the other hand there is not much of a difference between proximal and distal nerves. Later on at 37 days when two peaks have appeared in the muscle nerves their difference compared with the roots is even more striking. The peak of the biggest fibres in the medial gastocnemius nerve then lies at 6—7 microns which is about the same as for the adductor and the quadriceps. The peak

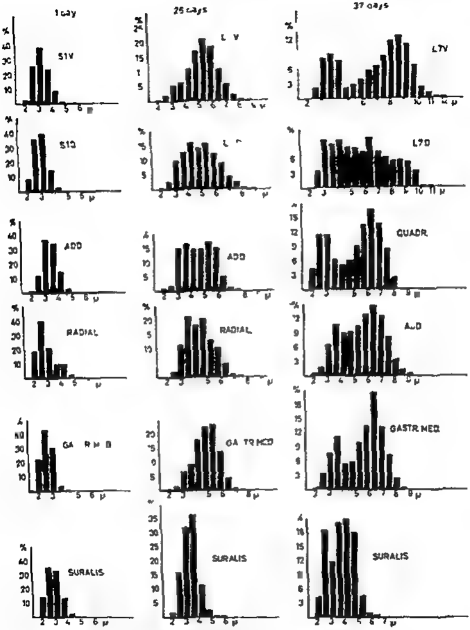


Fig 10 Histograms of ventral and dorsal roots and some muscle and skin nerves at different ages postnatally as given in the figure (For further explanation see text)

of the ventral root is then between 8—9 microns and both the ventral and the dorsal roots contain bigger fibres than the peripheral nerves. We can thus see a tendency of the roots to move ahead while the difference between the peripheral nerves becomes less and less. At 52 days (Fig. 11) consequently the peak for the big fibres in all muscle nerves lies around 7—8 microns as against 9—10 microns in the ventral root. At 91 days (Fig. 11) and 153 days (Fig. 12) this difference is even more pronounced. Still in the old grown animal (Fig. 1[illegible]) the roots have bigger fibres than the peripheral nerves.

The findings described above deserve a somewhat closer examination in some respects to become intelligible. Thus if we compare the spinal roots the dorsal roots are less advanced than the ventral ones and if we compare the amount of fibres between 1 and 3 microns we find a greater amount in the dorsal roots proportionally than in the ventral roots. This of course is explained by the well know fact that the ventral roots are myelinated before the dorsal ones (Ambron and Held 1895). Thus there are more unmyelinated fibres to become myelinated in the dorsal than in the ventral roots at birth. The same holds true for the peripheral nerves as compared with the roots because the process of myelination proceeds from the centre to the periphery (see Gutner 19[illegible]6). These findings are also reflected in the present material where it can be seen that there is proportionally more fibres between 1 and 3 microns in the dorsal and the mixed muscle nerves than in the ventral roots of the newborn animal where the peak is between [illegible]—4 microns. Thus there is a lagging behind of those fibres which will later on form the bulk of the large myelinated fibres in the dorsal root. This to some extent explains the difference between the peaks of the bigger fibres in the ventral roots and the mixed muscle nerves. As for the difference between the muscle nerves and the dorsal roots the same explanation can be applied because the process of myelination is a proximodistal affair. The peak however in the muscle nerves lies within the size range of the dorsal root but of course those fibres could be motor since we are dealing with a mixed nerve.

When different nerves are compared at the same age it is of great importance that they belong to one and the same animal. As can be seen from the Tables this conditions is fullfilled in most of the instances. However such comparisons must also be taken with great care especially in the younger age when the differences are small. As has been stated earlier by Skoglund (1960 b) the largest fibres in the peripheral nerves in the hind limb have [illegible]eached about the same size at birth and then they grow with

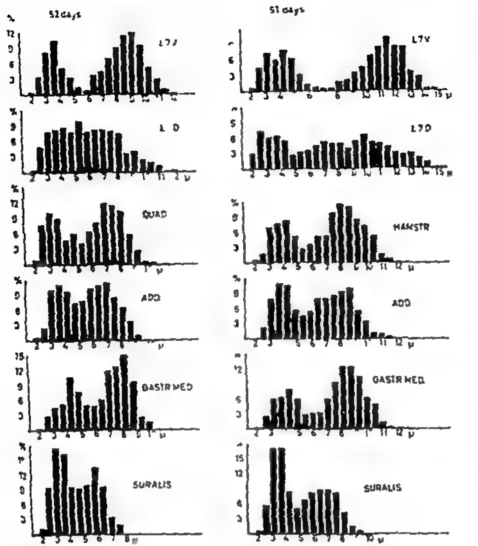


Fig 11 Histograms of ventral and dorsal roots and some muscle and skin nerves at two different ages (For explanation see text)

different speeds Although the differences are small they seem to be functionally important (see discussion)

In Fig 10 from a 1 day old animal (54 B) where the calibre spectrum from several nerves in the same animal are shown the sural nerve appears to have the same distribution of fibres as the medial gastrocnemius The Table however shows that this is only the case in this animal while in all others the muscle nerve is more developed There might be individual varia

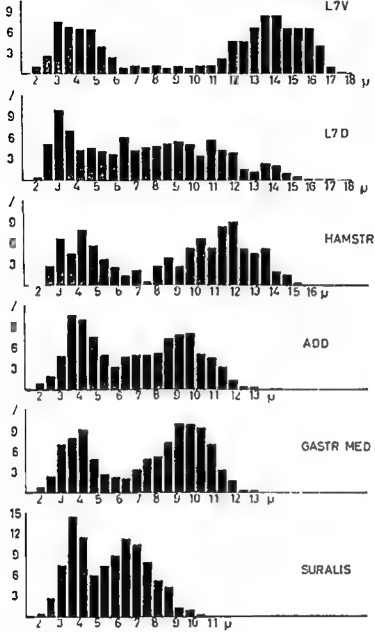


Fig 12 Histograms of ventral and dorsal roots and some muscle and skin nerves at 103 days postnatally (For further explanation see text)

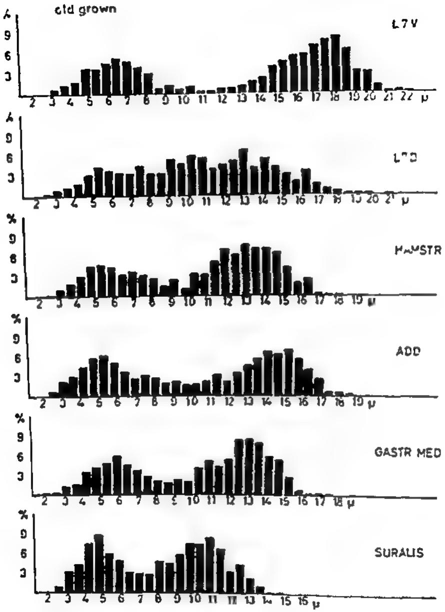


Fig 13 Histograms of ventral and dorsal roots and some muscle and skin nerves in the adult stage (For further explanation see text)

tions but a more likely explanation for this exceptional case is technical differences like the height at which the nerve was taken the histological treatment or errors of measurements

If we compare some of the calibre spectra in Fig 10 the observations that can also be extracted from the Tables are illustrated In the first place the dorsal and ventral roots are seen to have fibres of the same size but there are many more bigger fibres in the ventral root and relatively more small fibres in the dorsal root When comparing the ventral root with the nerve to the adductor it is seen that the adductor fibres are much bigger than in the S1 ventral root Unfortunately the upper lumbar roots could not be used to compare the adductor nerve with Comparing the adductor with the radial nerve shows that the adductor has as big fibres as the radial nerve in the forelimb and it is more developed than the medial gastrocnemius The differences between the medial gastrocnemius and the adductor is striking

The difference between the proximal and distal nerves as well as the differences between muscle nerves and roots which were dealt with above were noticed very early during the work on the postnatal development One reason for the differences could be that the nerve fibres are tapering and grow conically This has earlier been investigated by Reved (1944) and others in the phrenic nerve but they could not find any evidence for a conical growth In Table XVIII and XIX are found the calibre spectra of the saphenous nerve taken proximally in the inguinal region and distally at the height of the knee after the nerves to the knee joint have departed At a first look it appears as if there were bigger fibres in the proximal part of the nerve but a closer examination shows that the differences are extremely small Such differences do not allow us to draw any conclusions with regard to a possible conical growth of the nerve fibres

As pointed out earlier and shown in Fig 10 the radial nerve representing the cervical region and taken relatively distally at the elbow is more developed than the medial gastrocnemius nerve which represents a distal nerve in the lumbar region In Table XVI and XVII is presented the material on the radial and phrenic nerves respectively and it can be seen that they are as developed as the proximal nerves in the hindlimb If the same proximodistal development takes place in the forelimb which for physiological reasons (see discussion) is highly probable it should infer that the proximal nerves would be even more developed This is also supported by the known cephalo caudal development of the neuro axis (Kingsbury 1932)

evaluating the results presented it must be remembered that the ri on is based on the chronological age which as shown and discussed ,ur works (Skoglund 1960 a b c and d) might not be the best :e of maturity When dealing with comparisons in one and the same . all technical inconsistencies must be taken into consideration How ince all the results go in the same direction namely an increasing nce between the roots and the peripheral nerves investigated with sing age and a decrease of the difference between the same peripheral . with increasing age the slight variations in the early stages from :heme do not influence the general conclusions that there is a proxi tal development of the nerve fibre size These morphological results er can not because of the technical inconsistencies be directly cor :l with electrophysiological measurements of conduction velocity lund 1960 b) in which differences down to less than half a micron isily be ascertained It is striking though how well the two sets of s corre pond to each other Before discussing the physiological signifi of these morphological findings some of the results will be considered other viewpoints

the first place the differences in size between the largest fibres in mal and distal nerves is obvious This difference which is present in ewly born still prevail with regard to the roots and the peripheral s in the adult animal and might be due to a tapering of the fibres could not be ascertained The distance between the two sections of aphenous nerve used in this investigation might not be long enough above all measurements of fibres in groups of half microns might not re enough to reveal any differences Reyed (1944) could not find any nce for tapering either but the problem might be solved with electro iological measurements of conduction velocity The possibility also s that the reason for the difference between proximal and distal nerves e to more extensive branching in the latter during their longer course unly the nerves are branching as earlier shown by Eccles and Sherring 1930) but this can not be the whole explanation even if the branching s in the roots The evidence brought forward by Fernand and Young l) is not conclusive either These authors came to the conclusion that ching is not the sole explanation for the smaller fibres in the distal

nerves on account of the differences in number between proximal and distal sections of the same nerve They seem to overlook that the smaller fibres might branch too and by getting unmyelinated they are passing out of the spectrum since unmyelinated fibres can not be counted This easily explains their finding of a decrease in the largest size group of fibres from 30 to 4 over a length of 4 cm whereas the total number of fibres only increase from 305 to 317 Actually by serial sections of the spinal nerves it can be seen that the biggest fibres are leaving the trunk with the proximal nerves (Romero and Skoglund to be published) If the explanation for this difference depends upon that the innervation of proximal structures in a limb starts earlier than the innervation of distal ones can not be concluded (Fernand and Young 1951) Actually the difference between the roots and the distal nerves increases with increasing age (cf Fig 10 and 12)

Turning now to the correlation of these morphological findings with earlier physiological ones we will firstly point to the different speed of growth in cutaneous nerves as compared with muscle nerves This is in good agreement with the electrophysiological findings of Skoglund (1960 b)

Secondly there is found a difference in the maturity of proximal muscle nerves in comparison with distal ones in the younger ages This forms a good explanation for the finding (Skoglund 1960 c) that tonic responses from muscle spindles can be obtained earlier from proximal muscle than from distal ones Since the tonic response of the receptors can be correlated to the maturity of their nerve fibres (Skoglund 1960 c) The gradual appearance of posttetanic potentiation can also be correlated to the maturation of the fibres (Skoglund 1960 b Wilson 1962) In the light of the present findings this reaction should thus appear earlier in proximal than distal nerves Posttetanic potentiation has been considered a necessary mechanism for the appearance of tonic stretch reflexes (Granit 1957) and this is indirectly proven by the earlier appearance of tonic stretch reflexes from proximal muscles than from distal ones (Skoglund 1960 a) Thus in the newborn animal tonic stretch reflexes can be obtained from the pectoralis muscle innervated from the cervical region and this is in good accordance with the earlier development of the forelimb nerves as demonstrated here in the radial nerve Furthermore tonic stretch reflexes are obtained in newborn animal in the ilipsoas and adductor muscles in the hindlimb in good agreement with the finding of the earlier development of the adductor nerve

The postnatal development of the spinal roots can be correlated to the physiological finding regarding the gradual appearance of tonic stretch reflexes decerebrate rigidity and postural reflexes (Skoglund 1960 a) In

the light of the experimental finding that the nerve fibres attain adult electrical properties with regard to absolute refractory period and that tonic responses from muscle spindles and posttetanic potentiation can be shown at a conduction velocity of 15—20 m/sec (Skoglund 1960 c) corresponding to a diameter of 3—4 microns (Hursh 1939) it is interesting to see how the known functional development can be related to the morphological findings in the roots. The ventral roots in the newborn state have only one peak between 3 and 4 microns. This will later on separate into one peak between 6 and 7 microns and one between 2 and 3 microns 20 to 30 days after birth. After 10 days most of the large fibres have reached above 3 microns. Now we know that the motor nerves are functioning at birth and have developed further in the root at 10 to 20 days when the animal starts walking with its hindlegs. At that time the big motor fibres are well above 3 microns the peak lying at 5 microns in the root. One other mechanism that is of considerable importance in this connection for the development of sustained contractions in the hindlimb muscles is the posttetanic potentiation of the muscle twitch tension. This as shown by Nyström and Skoglund 1965 b can also be correlated to the maturation of the nerve fibres and has reached around 50 % of its adult value at the time the animal can walk.

Amongst the smaller fibers which later develop into a peak between 3 and 4 microns some of the gammafibres, which innervate the muscle spindles are to be found. These fibres must also become functionally mature before any tonic stretch reflexes can appear and before the animal can walk (see Skoglund 1960 a, b and c). The peak of these fibres develops very slowly and still at a 150 days it remains at 3—4 microns. This corresponds well to the electrophysiological findings that the gammafibres grow very slowly (Skoglund 1960 b).

Considering now the dorsal roots the tendency in the development is also clear. Here we have stated before that most of the fibres have not reached above 3 microns until after 20 days. In the light of the findings that the fibres are functionally mature at a conduction velocity of 18—20 m (3—4 microns) this corresponds quite well with the fact that the kittens can not walk and stand until three weeks and these abilities have not reached any perfectibility until around 40—50 days which in its turn corresponds to a well defined motor root with two peaks one gamma at 3—4 microns and one alpha at 8 microns and a dorsal root with the bulk of the large muscle afferents at 2—8 microns.

It is not our intention to push these correlations between the calibre spectra and the function too far. It is however quite a satisfactory corre

lation between morphology and function that has been achieved. It focuses the attention on the nerve fibre as one key to the understanding of the developmental mechanisms in the nervous system. Work, now in progress on the ultrastructure and histochemistry of the nerve fibre has given results that allow a further correlation between structure and function (Berthold and Skoglund 1965).

Summary

1. The postnatal development of spinal roots and peripheral nerves in the lumbosacral region is investigated in kittens from birth to 150 days old using osmium stained sectioned material. The results are compared with the adult animal and with nerves from the cervical region of the spinal cord.
2. It is found that the number of myelinated fibres increases during development being continually added to by the myelination of new fibres.
3. The development of the spinal roots is characterized by a greater maturity earlier in the ventral roots than in the dorsal roots. The ventral roots also develops faster than the dorsal ones. The development of the peripheral muscle nerves is still slower than that of the roots being even slower in the skin nerves.
4. Nerves to proximal muscles are earlier developed than those to distal muscles at birth but this difference decreases with increasing age. The opposite holds true for the relation between roots and muscle nerves in which a small difference at birth increases with increasing age. No explanation can be offered for the phenomenon.
5. The increase in the diameters of nerve fibres with age is characterized by a rapid growth in the beginning which gradually declines.
6. No evidence can be found for a tapering of the nerve fibres. This is discussed in the light of earlier results.
7. The findings are discussed in the light of earlier physiological work and correlated to the functional development of the nerve fibres.
8. A review of the earlier literature on postnatal development of number and size of nerve fibres is given.

This work is part of a series of investigations into the postnatal development of the nervous system supported by the Swedish Medical Research Council.

Table I The calibr

[illegible]	1 [illegible] 1 [illegible]	1 8 [illegible]	2. [illegible] 8	87 4	3 4 2 37	[illegible] 9 4 [illegible]	4 5 5 09	5 08 5 6.	5 [illegible] 6 18	6 18 6 7	6 [illegible]3 7 [illegible]5	[illegible]25 7.84	7 84 8 [illegible]	8 [illegible] 8 91	8 91 9 4[illegible]	9 49 10 04	10 04 10 60	10 60 11 15	11 15 11 70	11 7[illegible] 1
[illegible]	6	9	2	1[illegible]5	1	2														
A	.5	4[illegible]	[illegible]	223	63	8														
B	2	13	2 7	[illegible]27	93	[illegible]	1													
A	[illegible]3	1[illegible]	532	6	1 4	2		1												
A	28	1[illegible]	194	[illegible]0[illegible]	143	[illegible]9	.5	[illegible]1	6	1										
A	8	16	89	1.0	9	1 3	156	108	.	39	8									
A	3	1	59	1	60	6	91	58	9	58	4	.5	15	8	2			1		1
A	[illegible]	5	8	[illegible]	[illegible]4	57	51	61	60	[illegible]	41	2.	25	23	19	19	.5	14	5	8
1A	5	15	46	45	45	5	.1	61	.8	60	40	20	[illegible]	2.	[illegible]7		4	[illegible]		
A	9	4	43	25	23	[illegible]9	[illegible]1	1	.5	[illegible]	.3	27	[illegible]	[illegible]	31	16	41	8	4	[illegible]
A	4	.3	[illegible]3		3	34	.3	[illegible]	25	.6	.5	.5	[illegible]7	[illegible]5	20	32	4	6	5	24
A		[illegible]0	29	8	[illegible]1	60	5[illegible]	41	24	23	[illegible]5	85	[illegible]9	30	46	48	44	46	40	4
1A	4	8	4	22	25	41	21	.	9	5[illegible]	40	8	45	[illegible]	44	28	25	87	24	43

Table II The calibr

[illegible]	1 [illegible]3 1 6	1 0 .8[illegible]	2.2. 8	[illegible]8 .4	2 4. 3 9[illegible]	3 97 4 5.	4 5[illegible] 5 09	09 63	5 63 6 18	6 18 6 3	6 2 7 [illegible]	[illegible]9 .84	.84 8.29	8 29 8 91	8 91 9 40	9 40 10 04	10 04 10 60	10 60 11 1[illegible]	11 15 11 70	[illegible] 1.
4B	[illegible]	259	2 5	234	8[illegible]	14														
3A	[illegible]0	12[illegible]	2 4	[illegible]46	212	32														
4B	4.	167	248	817	1[illegible]0	11														
6A	22	95	110	1[illegible]1	103	5	4[illegible]	[illegible]	4	1										
A	[illegible]	145	120	100	1[illegible]0	127	65	4	10		1									
[illegible]A		14	40	160	1 6	19	[illegible]23	1[illegible]	20	10	2									
9A	[illegible]	[illegible]	.5	1	4	.9	21	[illegible]	4	8	[illegible]	8	6[illegible]	28	.3		1	1		
A	4	17	4	84	7	2[illegible]	87	4.	1	5	9	8	50	[illegible]	24	[illegible]	18	10	8	
1A	1	[illegible]9	[illegible]	73	2[illegible]	4	8	7	23	25	41	[illegible]	9	45	50	[illegible]	12	9	4	1
A	8	9	[illegible]	84	[illegible]	49	2[illegible]	[illegible]2	15	10	7	12	12	16	[illegible]1	[illegible]	31	50	84	[illegible]
A	2	18	[illegible]	2	2[illegible]	[illegible]0	3	[illegible]	4		7	8	8	16	16	[illegible]0	28	[illegible]	25	[illegible]
[illegible]A		1	6	10	2	2	42	50	.6	6.	21	2	23	2	[illegible]	3	2[illegible]	17	25	
[illegible]A			2	10	1.	23	[illegible]	42		41	41	25	23	1.	9	6	11		13	27

trum of the S 1 D

1 80–13 36	13 36–13 91	13 91–14 46	14 46–15 01	15 01–15 56	15 56–16 1	16 1–16 67	16 67–17 [illegible]2	17 [illegible]–17 77	17 [illegible]7–1 8	18 2–18 88	18 88–19 43	19 43–19 98	19 98–[illegible]53	[illegible]53–21 09	21 09–[illegible]1 64	1 64–[illegible] 19	[illegible]
1																	
9	1	1	1														
4	[illegible]	9	5	1	1												
41	6	1	15	14	9	7	5		1	1							
55	46	46	80	82	[illegible]1	19	[illegible]	11	6	1	2						

ectrum of the S 1 I

5–0	1 80–13 [illegible]	13 36–13 91	13 91–14 46	14 46–15 01	15 01–15 56	15 56–16 1	16 12–16 67	16 6–17.2	17 [illegible]–17 7	1 [illegible]7–18 [illegible]	18 32–18 81	18 84–19 45	19 43–19 95	19 94–20 3	[illegible] 1–1 0	21 04–21 [illegible]	1 [illegible]–[illegible] 19	[illegible]
	1	[illegible]	6	1	1													
	39	28	8	9	1													
	49	47	49	60	[illegible]	[illegible]	45	84	–	8								
	81	49	55	60	60	63	61	66	5	[illegible]6	15	12	8					

Table III The calibr

l	1 21 / 1 6	1 6 / ?3	2 2 / ?8	[illegible] / 3 42	3 4? / 3 97	3 97 / 4 5?	4 / [illegible]	5 08 / 5 63	5 63 / 6 18	6 18 / 6 3	6 3 / 5	7 ?8 / 7 84	?84 / 8 59	8 39 / 8 94	8 94 / 9 49	9 49 / 10 04	10 04 / 10 60	10 60 / 11 15	11 15 / 11 0	11 7 / 1
A	60	384	66	1?7	20	8														
B	[illegible]	1	02	?a	111	0	?													
A	4	..5	100	1	158	140	156	1 1	90	44	5	7	3							
A		..1	61	..6	?1	52	51	46	65	46	9	34	35	33	?1	5	2	?		
A	?	[illegible]	39	54	57	[illegible]	66	52	54	54	53	50	?5	27	15	13	[illegible]	1	8	
A	19	50	41	[illegible]	37	19	2	1	31	36	33	33	7	..8	45	35	3?	29	?1	18
A	?	3	7	51	50	3	?9	..6	44	31	33	35	39	40	37	5	41	31	?7	11
A	1	8	47		89	43	4	39	31	8	46	88	46	[illegible]	33	4	[illegible]	[illegible]	3	9
A		1	8	1?	1	30	4	37	33	3	44	31	83	58	47	59	66	83	46	[illegible]

Table IV The calibre

l	1 21 / 1 6	1 6 / ..32	2 / ?87	?8 / 3 4	3 4? / 3 97	3 97 / 4 5?	4 5? / 5 08	5 08 / 5 63	5 63 / 6 18	6 18 / 6 73	6 73 / 7 8	7 ?8 / 7 [illegible]	?84 / 8 39	8 ?9 / 8 94	8 94 / 9 49	9 49 / 10 04	10 04 / 10 60	10 60 / 11 15	11 15 / 11 70	11 7 / 12
3A	5	357	3?7	161	80	9	1													
4B	7	263	863	149	70	18		4	4											
A	1	1	55	66	105	167	207	186	107	66	?1	6								
0A		1	48	54	46	18	27	19	34	30	45	?9	6	[illegible]	40	[illegible]	7	1	1	
A	8	21	55	65		2	9	5	?3	?7	48	5?	71	4	6	8	19	7	?	
E	3	2?	[illegible]	28	39	31	16	5	4	8	5	9	11	13	3	34	4	51	4?	42
3A		13	38	88	8?	8	[illegible]	1	4	5	4	5	4	5	6	10	3	3	88	4
3A	8	2	12	54	46	54	[illegible]	67	[illegible]	41	33	19	19	[illegible]	4	8	8	10	10	18
?A			6	12	18	37	6	45	5	4	40	30	6	11		11	4	8	7	8

Table V The calibre

al	1 21 / 1 6	1 6 / 2 2?	5 / ?6	2 87 / 3 4	3 42 / 3 97	3 9 / 4 5	4 5 / 5 08	5 08 / 5 63	5 63 / 6 18	6 18 / 6 73	6 73 / 7 ?8	7 ?8 / 7 84	7 84 / 8 39	8 39 / 8 94	8 94 / 9 49	9 49 / 10 04	10 04 / 10 60	10 60 / 11 15	11 15 / 11 0	11 70 / 1
3A	[illegible]	?01	2 3	183	0	4														
4B	[illegible]	306	311	199	65	5														
A		3	97	01	??1	168	108	6	[illegible]	2	[illegible]	3								
0A		9	?9	6?	63	8	7	62	0	59	1	41	14	14	4	8	2			
3A	3	?0	58	45	1	64	..3	59	68	47	43	8	?4	[illegible]	11	10				
2A	1	[illegible]	51	?3	9	27	..3	5	8	86	35	39	9	27	37	84	?4	?5	19	15
3A	9	38	?	?4	49	85	[illegible]	1	?6	?9	2?	2	2	31	?8	..3	30	29	6	10
5A	7	31	66	6	4	5	9	37	28	8	4	51	40	31	..4	37	33	83	[illegible]	39
9A	4	14	31	30	30	34	..6	83	86	[illegible]	54	8	4?	9	43	45	37	31	43	47

of the L 7 D

	13 36–13 91	13 91–14 46	14 46–15 01	15 01–15 56	15 56–16 1	16 12–16 6	16 6–17 [illegible]	1 [illegible]–1 77	1 7–18 [illegible]	18 [illegible]–18 8[illegible]	18 88–19 43	19 43–19 98	19 94–[illegible]	[illegible]	1 08–1 64	1 61–[illegible] 19	[illegible] 12–[illegible] 4	Total fibres measured
																		1 000
																		1 000
																		1 000
																		6.0
																		[illegible]
	9	1																690
	15	8	4	1	1	1												80
	83	[illegible]	[illegible]	1	9	[illegible]	11	13	4	3	1							1 000
	53	43	80	20	86	16	10	7	8	2	2		1					1 000

a of the L 7 I

	13 86–13 91	13 91–14 45	14 46–15 01	15 01–15 56	15 56–16 1	16 12–16 6	16 67–[illegible] 22	1 [illegible]–17 77	1 [illegible]–18 [illegible]	[illegible]–18 8[illegible]	18 8[illegible]–19 48	19 43–19 9[illegible]	19 96–[illegible] 53	[illegible] 53–1 08	[illegible]1.0[illegible]–21 64	1.64–[illegible] 19	[illegible]–[illegible] 74	Total fibres measured
																		1 000
																		1,000
																		1 000
																		600
																		[illegible]
	2	2																500
	83	83	[illegible]	5	2													616
	43	[illegible]	60	[illegible]	55	56	[illegible]	40	[illegible]	5	2	1						1 000
	[illegible]	9	45	51	56	69	77	8	61	21	31	12	[illegible]	4	1			1 000

n of the L 6 D

	[illegible]	13 86–13 91	13 91–14 46	14 46–15 01	1 [illegible]–15 6	15 56–15 12	16 1–16 67	16 6[illegible]–17.2[illegible]	1 [illegible]–1 77	1 7–18 3	18 3–18 88	18 88–19 43	19 43–19 93	19 [illegible]–[illegible]0 53	[illegible]0 53–[illegible]1 03	[illegible]1 03–1 64	[illegible]1 64–[illegible] 19	[illegible] 19–[illegible] 4	Total fibres measured
																			800
																			1 000
																			1 000
																			640
																			[illegible]
		9	8																[illegible]
		11	4	8	1														700
		37	[illegible]3	19	1	20	11	9	7	2									1 000
		85	80	26	20	18	21	3	1	11	6	4	–						1,000

Table VI The

	Animal no.	1.21 1.6	1.6	2.87	2.57 3.49	3.49 3.97	3.9 4.59	4 5.09	5.09 5.63	5.63 6.18	6.18 6.3	6.3 7.28	8 7.84	[illegible] 8.9	8.9 8.91	8.91 9.49	9.49 10.04	10.04 10.60	10.60 11.15	11
	[illegible] A	1	206	43	150	41	7													
	84B	19	1 6	401	239	9	[illegible]													
	4 [illegible] A	4	90	[illegible]	61	111	97	67	184	0	70	8	3	1						
	15[illegible] A	1	[illegible]	73	[illegible]	19	15	73	0	66	84	94	[illegible]	55	51	10	5			
	1[illegible]2A	5	5	[illegible]	55	23	31	8	15	29	[illegible]	40	[illegible]	7	66	51	[illegible]	8		1
	1[illegible] A	4	1	90	22	1	16	10	10		6	1	4	5	7	17	[illegible]	8		88
	1[illegible] A	1	6	34	49	4	4	17	9	3	[illegible]		1	5	7	8	18	18	73	36
rg n	4 A		6	23	49	[illegible]	52	6[illegible]	[illegible]	5	6	24	18	4	9	4	7	7	9	19
1 wn	468A	1	[illegible]	11	14	26	73	[illegible]	[illegible]	56	[illegible]	40	29	1	10	2	7	4	7	[illegible]

Table VII The

	Animal no.	1.21 1.76	1.6	2.8 6	6 4	3.4 3.9	3.9 4.5	4.5 5.09	5.09 5.63	5.6 6.18	6.18 6.3	6.73 7.28	7.84	7.84 8.39	8.9 8.94	8.94 9.49	9.49 10.04	10.04 10.60	10.60 11.15	11
	[illegible]8A	5	1 1	44		2														
	4 A	3	[illegible]	163	191	199	146	119	71	22	73	8	6							
	160A	9	36	[illegible]	85	[illegible]	[illegible]	68	4[illegible]	44	2[illegible]	[illegible]	1[illegible]	19	8	3		[illegible]		
	161A	13	63	[illegible]	50	66	6	0	41	9	79	7	19	25	10	6	8	2		
	1[illegible] A	6	[illegible]	49	4[illegible]	43	[illegible]	40	80	[illegible]	[illegible]	35		49	7	89	[illegible]	76	[illegible]	[illegible]
	1[illegible]9A	7	4	61	3	44	41	2[illegible]	79	[illegible]	23	23	[illegible]	[illegible]	13	79	[illegible]	15	1	11
ung	46[illegible]A	1	58	[illegible]	187	99	5[illegible]	60	49	8[illegible]		1	34	25	41	5	6	26	[illegible]	48
1	4[illegible]8A	1	11	[illegible]		[illegible]	81	61	49	46	5	4[illegible]	4	4	48	40	[illegible]	[illegible]	54	[illegible]

Table VIII The

	Animal no.	1.21 1.6	1.6 [illegible]	[illegible] 87	2.8 3.4	2.4 [illegible]9	9 4	4 [illegible]09	5.0 5.64	5.6 6.18	6.18 6	6.3 [illegible]8	[illegible] 84	8.4 8.9	8.9 8.91	8.91 9.49	9.49 10.04	10.04 10.60	10.60 11.15	11
	53A	40	4	13	61	4														
	[illegible]4B	79	110	16	179	6	8	1												
	4 [illegible]A	1	[illegible]	1	110	110	160	15	143	12[illegible]	[illegible]	73	7	4	1					
	160A	4	9	4	6[illegible]	90	[illegible]	45	46	2	4	69	41	47	1[illegible]	5	[illegible]			
	1[illegible]1A	4		104	79	1	11	15	[illegible]	41	4	64	69	[illegible]	76	11	5	[illegible]		
	16 [illegible]	9	4[illegible]	9	[illegible]	9	[illegible]	[illegible]	19	13	9	6	9	9	15	25	2[illegible]	2	30	9
	63A		31	66	65	79	21	1	10	1		3			6	1	75	73	30	39
wn	4 A	[illegible]	[illegible]	90	1	49	69	60	68	[illegible]		1	10	6	4	2	5	10	7	15
ing wn	4[illegible]A	[illegible]	123	129	99	1	53	[illegible]	23	43	13	9		0	7	8	11	10	19	18

L 6 1

… 01 … 46	11 46 15 01	15 01 15 50	15 .4 16 1	16 1 16	16 17	17 17	1 7 1 3	18	18 88 1 43	19 43 19 9	19 9 0 5	0 53 1 0	1 0 1 6	21 6 1	2 19	[illegible]
																[illegible]
																[illegible]
																1 000
																610
																[illegible]
	1															[illegible]
	16	11	5	2												[illegible]
	43	65	[illegible]	4[illegible]	[illegible]	55		4	1							[illegible]
	[illegible]	61	45	58	[illegible]	5	4	46	8[illegible]	1	11	5	[illegible]		2	1 [illegible]

e I o D

13 91 14 46	14 46 15 01	15 01 15 56	15 56 16 1	16 1 16 6	16 67 17 2	1 1	17 7 1	18 2 1	18 88 17 43	19 43 19 94	19 9	0 53 1 0	1 0 1 6	1 64 2 19	19	[illegible]
																[illegible]53
																1 0[illegible]1
																612
																[illegible]20
	8	1														[illegible]90
[illegible]	2	2	2													[illegible]30
10	5	5	[illegible]	3	1	1	1									1 100
17	17	10	5	3	16	8	6	3	3							1 000

he L 5 1

13 91 14 46	14 46 15 01	15 01 15 56	15 56 16 1	16 1 16 6	16 67 17	17.2 17 7	1 18 82	18 2 19 88	18 88 19 43	19 43 19 94	19 94 0 53	0 1 0	1 0 1 61	1 61 2 1	19 74	[illegible]
																5[illegible]0
																450
																1 000
																610
																640
7	1															590
6		1	2	1												510
4	48	58	45	52	[illegible]	39	[illegible]	16	10	3						1 000
17	8	7	4	18	15	13	4	4								1 000

Table IX The calibre

1 1 1 1 6 2	5 [illegible]	2 2 2 [illegible]	[illegible] 3 4	2 42 3 9[illegible]	2 [illegible] 4 32	4 5 08	5.09 5 [illegible]3	5 [illegible]8 6 18	6 18 6 3	6 3 28	7 28 84	7 84 8 [illegible]9	[illegible] 9 8 94	8 91 9 4[illegible]	9 49 10 01	10 01 10 60	10 60 11 15	11 15 11 [illegible]0	11 70 1[illegible] 25
8	1 9		10	1															
11	45	[illegible]	[illegible]	1 2	[illegible]	94	71	21	8	[illegible]									
8	49	[illegible]	90	[illegible]	67	[illegible]	46	41	28	23	1	6	5	4	8				
18	[illegible]	18	[illegible]	6	[illegible]	53	47	45	[illegible]	[illegible]	20	[illegible]	17	[illegible]	5	6	1		
28	6	61	[illegible]	[illegible]	6	[illegible]	[illegible]	[illegible]	[illegible]	23	21	[illegible]	23	88	[illegible]	26	14	9	1
2	31	40	[illegible]	[illegible]	74	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	25	16	14	2	8	16	[illegible]		8
8	28	124	[illegible]	[illegible]	54	[illegible]	53	43	[illegible]	89	[illegible]	28	[illegible]	[illegible]	[illegible]	[illegible]	85	80	25
1	[illegible]	[illegible]	[illegible]	43	47	40	57	58	51	42	88	28	48	46	44	[illegible]	47	86	[illegible]

Table X The calibre

1 21 1 [illegible]	1 [illegible] 2 [illegible]	[illegible] 8[illegible] [illegible]87	2 8 5 4[illegible]	[illegible]42 3 97	8 [illegible]7 4 5[illegible]	4 5 5 05	5 05 5 [illegible]	5 63 6 16	6 18 [illegible] 78	6 3 28	7 28 84	7 84 8 [illegible]	8 32 8 [illegible]	8 91 [illegible]49	9 43 10 01	10 01 10 60	10 60 [illegible] 1	11 15 11 70	11 70 12 [illegible]5
18	68	65	[illegible]	14	4														
1	11	29		123	[illegible]	[illegible]	[illegible]	[illegible]	10	2									
8	[illegible]	[illegible]	48	29	81	68	101	95	6	27	[illegible]	8	2	2	1				
3	68	73	44	27	19	9	[illegible]	21	23	89	6	[illegible]	[illegible]	14	6	8			
[illegible]	141	125	5	53	2	[illegible]	8	3	8		4	4	[illegible]	6	14	16	[illegible]	[illegible]	[illegible]
8	[illegible]	4	1	25	25	9	2	6	8	8	10	10	13	13	14	20	22	[illegible]	28
[illegible]	[illegible]	[illegible]	114	[illegible]	65	28	23	[illegible]	30	10	6	9	7	8	7	11	11	19	19
11	8	[illegible]	83	8		[illegible]	41	21	18	11	8	8	4	6	9	7	12	16	[illegible]

Table XI The calibre

1 21 1 [illegible]	1 6 2	2 [illegible] [illegible]	2 87 3 4	8 42 3 9[illegible]	8 97 4 52	4.[illegible] 5 05	5 04 5 6[illegible]	5 63 6 18	6 18 6 3	6 3 28	7 8 [illegible]4	84 8 29	8 32 8 91	8 94 9 4[illegible]	9 43 10 01	10 01 10 60	10 60 11 1	11 15 11 70	11 0 12 5
6	68	[illegible]	[illegible]	[illegible]	15														
6	[illegible]	143	181	106	63	1													
7	56	1 0	110	102	[illegible]	25	8												
1	8	110	[illegible]	91	81	[illegible]	[illegible]	17											
		[illegible]	[illegible]	[illegible]	48	2	70	60	45	20	6								
4	9	3	81	71	1	84	3	[illegible]	5	[illegible]									
9	41	[illegible]	3	[illegible]	[illegible]	1	68	60	41	[illegible]	15								
3	9	[illegible]	60	[illegible]	61	6	1	81	[illegible]	4	19		2						
2	12	[illegible]	[illegible]	51	[illegible]	41	5	[illegible]	50	44	[illegible]	[illegible]	5	4					
	10	7	12	[illegible]	[illegible]	1	[illegible]	28	28	22	41	49	26	16	5	4	1		
[illegible]	10	[illegible]	[illegible]	[illegible]	27	37	36	[illegible]	[illegible]	[illegible]	29	45	28	43	[illegible]	[illegible]	11	5	4
1	[illegible]	16	[illegible]	23	[illegible]	5	9	4	7	10	15	18	1	[illegible]	24	24	20	9	5
5	10	[illegible]	[illegible]	[illegible]	4	29	[illegible]	8	[illegible]	29	[illegible]	44	47	48	[illegible]	27	19	7	2
[illegible]	4	23	[illegible]	28	41	[illegible]	4	[illegible]	[illegible]	21	17	[illegible]	14	[illegible]	10	17	16	23	22
	4	15	20	[illegible]	[illegible]	4	25	[illegible]	19	21	18	18	[illegible]	[illegible]	12	14	[illegible]	16	21

spectrum of the L4D

1 5 12 80	1 80 13 36	13 36 13 91	13 91 14 46	14 46 15 01	15 01 15 56	15 6 16 1	16 12 16 67	16 6 17.22	17.2 17	17 7 18.27	18 8 18 88	18 88 19 43	19 43 19 98	19 98 20 53	20 53 21 08	1 08 1 [illegible]	1 64 19
9	7	6	1	2			1										
5	3		1														
19	15	14	5	8	6	5	2	1									
28	23	12	12		2	3	1	2									

spectrum of the L4V

12 .5 1 80	1 80 13 36	13 36 13 91	13 91 14 46	14 46 15 01	15 01 15 56	15 56 16 12	16 1 16 67	16 67 17.2	1 [illegible]	17 77 18 8	18 8 18 88	18 88 19 43	19 43 19 98	19 98 20 53	20 53 21 08	21 08 21 64	1 64 19
6	24	23	8	1	1												
34	21	21	8	8	8	4	2										
11	20	23	15	5	4	4	1	6	7	2	1			1			
15	28	8	29	36	38	41	40	35	6	16	11	4	1				

spectrum of the Adductor Nerve

1 .25 12 80	1 80 13 36	13 36 13 91	13 91 14 46	14 46 15 01	15 01 15 56	15 56 16 12	16 12 16 6	16 67 17 2	1 17 7	17 18 8	18 8 18 88	18 88 19 43	19 43 19 98	19 98 20 53	20 53 21 08	21 08 21 64	21 64 19
1																	
41	41	45	39	[illegible]	18	17	4	2	1	1							
31	38	[illegible]	45	48	[illegible]	[illegible]	18	4	4	2							

Table XII The calibre

1 1 / 1 6	1 6 / 8	2 / 87	87 / 3 4	3 4 / 8 97	8 / 4 5	4 5 / 5 08	5 08 / 5 63	5 6 / 6 18	6 18 / 6 73	6 / 3	[illegible] / 7 84	84 / 8 39	8 9 / 8 94	8 94 / 9 49	9 49 / 10 04	10 01 / 10 60	10 6 / 11 15	11 1 / 11 0	11 0 / 1
81	155	110	13	1															
31	181	196	80	1															
13	107	1 0	195	0	5														
3	47	3	7	6	6	100	49	26	3										
1	1	69	91	1 6	24	33	138	45	6	2									
[illegible]	18	41	60	30	3	54	3	113	[illegible]	40	[illegible]	[illegible]							
2	[illegible]	0	6	53	[illegible]	76	1 1	12	87	6	0	6	1	1					
	17	8	3	60	48	81	0	87	3	8	93	59	16	10					
1	14	8	88	43	3	15	17	19	3	51	68	68	57	31	6		1		
4	1	9	45	47	5	15	14	10	24	7	35	87	[illegible]	50	8	83	16	13	[illegible]
4	14	4	46	55	29	15	14	1	1	28	30	43	60	58	57	4	19	10	2
1	8	8	11	11	13	[illegible]	80	90	28	8		80	[illegible]	8	40	9	[illegible]	87	[illegible]
1	2	8	10	4	28	33	40	31	6	19	14	13	16	14	5	85	88	86	59

Table XIII The calibr

1 1 / 1 6	1 76 / 8	8 / 87	8 / 2 42	3 4 / 3 97	97 / 4 5	4 5 / 5 08	5 08 / 5 63	5 3 / 5 18	6 1 / 6	6 5 / 7 8	7 3 / 84	7 84 / 8	8 39 / 8 94	8 94 / 9	1 49 / 10 04	10 04 / 10 60	10 60 / 11 15	11 15 / 11 0	11 / 1
6	1	159	47																
1	161	203	181	41															
8	[illegible]	86	77	85	160	171	104	41											
8	8	44	9	134	168	1	5	13	4										
17	5	10	4	63	6	183	1	114	87	1	4	1							
3	6	43	40	39	68	104	10	9	51	31	15	8	2						
17	9	100	6	100	10	115	1 0	95	60	13	1	1							
1	23	49	54	45	29	17	87	78	81	93	6	21	7	1					
1	7	41	68	41	9	17	1	31	60	65	9	86	60	41	10	6			
15	81	40	40	83	3	[illegible]	20	5	19	[illegible]	23	48		8	4	86	15	5	
3	7	41	41	31	29		10	[illegible]	1	19	6	27	86	85	5	9	29	18	4
1	0	19	4	[illegible]	2	4	81	38	60	0	9	8	13	[illegible]	2	86	8	46	51
8	17	18	16	17	[illegible]	43	83	[illegible]	39	8	18	13	3	1	6	44	40	0	68

Table XIV The calibr

1 21 / 1 6	1 6 /	/ 8	/ 3 4	3 4 / 97	3 7 / 4 5	4 5 / 5 08	5 09 / 5 63	5 3 / 6 18	6 18 / 6 73	6 3 /	8 / 84	7 84 / 8 89	8 89 / 8 94	8 94 / 9 4	9 4 / 10 04	10 04 / 10 60	10 0 / 11 15	11 15 / 11 0	11 / 1
17	1 5	0	[illegible]	190	1 1	58	81	11	5	8	1								
1	9	69	116	113	69	239	118	[illegible]	8	1									
3	45	46	[illegible]	24	8	49	8	31	5	46	12								
5	46	60	53	81	6	24	[illegible]	49	71	68	6	85	16	3	1				
	16	59	46	3	1	30	8	60	2	9	[illegible]	49	8	11	4	1	1		
1	17	57	53	4	[illegible]	6	13	[illegible]	1	10	20	[illegible]	46	8	60	79	[illegible]	8	
	2	[illegible]	83	41	66	57	67	41	4	83	27	1	[illegible]	38	48	43	41	61	56
		3	11		49	4	49	59	[illegible]	8	31	19	2	2	[illegible]	[illegible]	[illegible]	59	6

spectrum of the Gastrocnemius Med

1..25 1 .80 13.36 13 91 14 46 15 01 15 56 16 12 16 6 1 .. 17 7 18 32 18 84 19 43 19 94 .0 53 .1 04 .1 64 .. 1.
.. 80 13 36 13 91 14 46 15 01 15.56 16 1 16 6 17 .. 17 18 32 18 8 19 43 19 94 .0 53 1 .8 21 64 .. [illegible] ..

1
22 14 9 6 8 * 1 2 1 1
9 53 40 85 19 5 1 2 1

spectrum of the Gastrocnemius Lat

1...5 12 80 13 36 13 91 14 48 15 01 15..6 15 1 16 6 1 2. 1 .. 18.2 18 84 19 43 19 94 .0 53 1 04 1 64 .. 1
1 80 13 .6 13 91 14 46 15 01 15 56 16 12 16 67 17 .. 1 .. 18 32 18 84 19 43 19 .4 .0 .3 1 04 1 64 .. 19 ..

89 46 [illegible] 63 44 [illegible] 26 1 8 2 2 1
64 56 43 34 36 9 2 1 1

spectrum of the Quadriceps Nerve

1.. 5 1 80 13 3. 13 91 14 46 15 01 15..6 16 12 16 6 1 .. 1 .. 18 8 18 85 19 43 19 93 20 53 1 08 1 64 ..
1. .0 13 .6 13 91 14 46 15 01 15.5. 16 12 16 67 1 .. 17 77 18 8. 18 85 19 43 19 93 .0 53 .1 08 21 64 22 19 ..

1 5 3
46 [illegible] 40 [illegible] [illegible] 19 [illegible] 8 1 2 1
4 [illegible] 51 50 [illegible] 24 15 2 1

1.71 1.6	1.6 [illegible]	8 [illegible]	[illegible] 4	[illegible] 2.89	2.89 4.5	4.5 5.05	5.05 5.63	5.63 6.18	6.18 6.73	6.73 7.28	7.28 7.84	7.84 8.39	8.39 8.94	8.94 9.49	9.49 10.04	10.04 10.60	10.60 11.15	11.15 11.70	11.70 12
9	[illegible]	13	150	[illegible]	[illegible]	1.4	45	5		3	1								
	[illegible]	43	1	15	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]										
		[illegible]	[illegible]	63	6	[illegible]	[illegible]	[illegible]	90	61	[illegible]	8	4						
9	[illegible]	5	6	41	54	[illegible]	[illegible]	[illegible]	[illegible]	2	22	9	[illegible]						
	8	7	41	[illegible]	[illegible]	14	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	60	46	29	8	8	4		
	16	29	[illegible]	4	23	[illegible]	[illegible]	[illegible]	1	[illegible]	16	[illegible]	15	[illegible]	[illegible]	21	49	5	80
		5	[illegible]	22	27	46	54	5	2	[illegible]	[illegible]	18	17	15	[illegible]	[illegible]	[illegible]	9	49
	1	10	18	20	41	4	[illegible]	[illegible]	[illegible]	3	[illegible]	15	[illegible]	[illegible]	21	[illegible]	5	0	61

Table XVI The calibre

1.71 1.6	1.6 [illegible]	[illegible]	[illegible]	[illegible] 2.89	2.89 4.5	4.5 5.05	5.05 5.63	5.63 6.18	6.18 6.73	6.73 7.28	7.28 7.84	7.84 8.39	8.39 8.94	8.94 9.49	9.49 10.04	10.04 10.60	10.60 11.15	11.15 11.70	11.70 12
10	40	[illegible]	60	[illegible]	[illegible]	1													
[illegible]	[illegible]	[illegible]	1.5	59	[illegible]	1													
54	40	16	66	44		[illegible]	19	5	1										
	16	11	[illegible]	150	[illegible]	123	101	41		[illegible]									
	[illegible]	61	61	45	4	[illegible]	41	45	5	[illegible]	6	50	29	1	[illegible]	1	1	1	
4	16	9	46	[illegible]	51	[illegible]	89	19		80		41	[illegible]	47	[illegible]	[illegible]	[illegible]	8	4

Table XVII The calibre

1.71 1.6	1.6 [illegible]	[illegible]	[illegible]	[illegible] 2.89	2.89 4.5	4.5 5.05	5.05 5.63	5.63 6.18	6.18 6.73	6.73 7.28	7.28 7.84	7.84 8.39	8.39 8.94	8.94 9.49	9.49 10.04	10.04 10.60	10.60 11.15	11.15 11.70	11.70 12
14	1.4	190	[illegible]	[illegible]	4														
[illegible]	143	[illegible]	140	46	[illegible]														
8	[illegible]	3	[illegible]	14	120	0	1	[illegible]											
1	11	[illegible]	[illegible]	[illegible]	[illegible]	108	[illegible]	[illegible]											
4	[illegible]	[illegible]	15	[illegible]	4	65	[illegible]	105	6	80	[illegible]	16	6						
	[illegible]	[illegible]	16	19	26	61	[illegible]	[illegible]	4	2	41	[illegible]		15	[illegible]		1		

Table XVIII The calibre

1.71 1.6	1.6 [illegible]	[illegible]	[illegible]	4 [illegible]	2.89 4.5	4.5 5.05	5.05 5.63	5.63 6.18	6.18 6.73	6.73 7.28	7.28 7.84	7.84 8.39	8.39 8.94	8.94 9.49	9.49 10.04	10.04 10.60	10.60 11.15	11.15 11.70	11.70 12
15	15	[illegible]	115	4															
20	[illegible]	[illegible]	1	4	14	2													
[illegible]	[illegible]	11	110	[illegible]	99	[illegible]	2												
6	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	4	1												
8	4	[illegible]	[illegible]	122	9	4	[illegible]	[illegible]	2										
3	63	91	65	[illegible]	[illegible]	[illegible]	51	[illegible]	9	2									
15	[illegible]	171	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	91	60	41	[illegible]	[illegible]	1	1				
6	48	[illegible]	[illegible]	101		[illegible]	[illegible]	[illegible]	5	58	[illegible]	[illegible]	15	4	4				
11	[illegible]	11	120	[illegible]	[illegible]		49	61	6	5	6	48	43	[illegible]	6	1			
1	4	[illegible]	65	121	99	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	61	1		[illegible]	[illegible]		[illegible]	5	[illegible]

spectrum of the Hamstring Nerve

12.. 12 90 13 3[illegible] 13 [illegible] 14 46 15 01 15 [illegible] 16 1[illegible] 16 [illegible] 1 [illegible] 1 [illegible] 1[illegible] 1[illegible] 19 43 19 9 [illegible]
1 [illegible] 13 .[illegible] 13 81 [illegible] 46 15 01 15 56 16 12 16 67 1 [illegible] 1 — 1 [illegible] 1 [illegible] 19 43 19 94 [illegible]

.5 [illegible] 11 9 1
65 6[illegible] [illegible] 0 63 [illegible] 36 [illegible] 1 [illegible] [illegible] [illegible]
6 71 65 43 [illegible] .5 4 2

spectrum of the Radial Nerve

1 5 12 80 13 86 13 81 14 46 15 01 15 [illegible] 16 12 16 [illegible] 1 [illegible] 1 [illegible] 19 [illegible] 19 [illegible] 19 43 19 9 [illegible]
1 80 13 .6 13 81 14 46 15 01 15 56 16 1 16 [illegible] 17 [illegible] 1 [illegible] 19 82 1 [illegible] 19 44 19 9[illegible] [illegible]

1

spectrum of the Phrenicus Nerve

12 .5 1 80 13 86 13 81 14 4[illegible] 15 01 15 56 16 1 16 [illegible] 1 [illegible] 1 [illegible] 19 2 1[illegible] 19 43 19 9[illegible] [illegible]
1..80 13 86 13 81 14 46 15 01 15 [illegible] 16 12 16 [illegible] 17 [illegible] 1 [illegible] 19 82 19 [illegible] 19 43 19 [illegible] [illegible]

spectrum of the Saphenus Nerve Prox

1 1.. 80 13 3[illegible] 13 81 14 46 15 01 15 56 16 12 16 6 1 [illegible] 1 [illegible] 18 82 18 8[illegible] 19 43 19 09 20 .3 [illegible]
1 80 13 33 13 81 14 46 15 01 15 56 16 1 16 6 1 [illegible] 1 [illegible] 19 2 19 88 19 43 19 99 [illegible]

Table XIX The calibre

Age in days	Animal	1 7? 1 ?	1 6 3	?	2 87 3 4?	3 42 3 9	3 97 4 5?	4 5 5 04	5 08 5 63	5 63 6 18	6 19 6 3	6 3 ?	9 5?	7 24 8 3?	8 39 8 91	8 91 9 4?	9 49 10 01	10 04 10 60	10 60 11 15	11 15 11 0	11 0 12 ?
10	40A	80	105	160	90	8?	5	3													
11	5A	[illegible]	148	131	195	51	8	5	5												
9	47A	80	193	159	113	4	13														
6	4 A		58	6	?58	143	8?	3													
8	100A	1	135	107	101	119	90	?3	?	?											
[illegible]	101A	13		151	61	103	50	64	85	17	?										
91	1? A		8	191	100	83	61	10?	?0	85		7	9	8		2					
133	103A	6	41	101	1 0	91	[illegible]	4	60	85	1	50	1	65	7?			[illegible]			

Table XX The calibre

Age in days	Animal	1 1 1 6	1 0 5?	? ?8	8 8 4	3 42 3 9	3 9 4 5	4 2 5?9	5 08 5?9	5 69 6 18	6 18 6 3	6 3 ?	8 5?	84 8 9	8 81 8 91	8 91 9 49	9 49 10 01	10 04 10 60	10 60 11 15	11 15 11 0	11 0 1 3
1	81B	44	10	100	42																
4	83B	81	191	L	44	4															
	D169B	0	159	100	5	2															
7	D163B	[illegible]	250	171	80	1															
11	78A	63	?10	1 8	191	[illegible]	8	1													
5	4 A	15	157	1 7	167	115	8														
?6	14 A	4	40	80	90	9	6	1	1												
6	D1 A	10	[illegible]	80	43	7	4														
85	100D	1	1 5	10	8	155	1	8	0	4											
2	101A	15	61	41	7	6	63	[illegible]	3	2											
42	11 C	6	[illegible]	1	95	95	1 3	8	31												
4	D155C	[illegible]	1	1 7	101	100	119	8	0	8											
	103A		[illegible]	1 5	10	63	61	[illegible]	28	63	1	12									
	16 A	4	45	15	13	[illegible]	[illegible]	54	66	71	71	6?	25	12	6						
	103A		15	41	6	79	58	41	55	69	6	4	1	6	[illegible]	8	1				
[illegible]	106A	4	5		15	[illegible]	2	[illegible]	35	30	5	50	41	56	53	31	40	51	[illegible]	10	8
1	469A	5	1	8	43	56	35	87	0	19	18	31	28	9	49	43	[illegible]	44	1	29	15

...um of the Saphenus Nerve distalis

80	13 36	13 91	14 46	15 01	15 56	16 1	16 6	17 [illegible]	[illegible]	18 3	18 84	19 43	19 94	[illegible] 53	21 04	1 61	[illegible] 19
.36	13 91	14 46	15 01	15 [illegible]	16 1	16 67	17 22	1 7	18 3	18 84	19 43	19 95	[illegible] 53	1 04	1 61	[illegible]	[illegible] 74

...um of the Sural Nerve

2 80	13 36	13 91	14 46	15 01	15 56	16 12	16 6[illegible]	[illegible]	1 7	18 32	18 88	19 43	19 94	[illegible] 53	1 04	1 6[illegible]	[illegible] 19
3 36	13 91	14 46	15 01	15 56	16 1	16 67	[illegible]	17 7	18	18 88	19 43	19 94	[illegible] 53	1 [illegible]	1 61	[illegible] 19	[illegible] 4

1		1
1	1	

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ACTA PHYSIOLOGICA SCANDINAVICA
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THE IMPULSE ACTIVITY IN DIFFERENT PARTS OF THE SLOWLY ADAPTING STRETCH RECEPTOR NEURON OF THE LOBSTER

BY
WOLFGANG GRAMPP

GÖTEBORG 1966

RESULTS

The conduction of the active process along the membranes of different cell regions

Firing with single spike discharges

Extracellular recording The spike activity of the slowly adapting stretch receptor nerve cell which was kept in a large volume of conducting fluid could be registered with extracellular microelectrodes which were placed close to the nerve cell membrane When recording at different cell regions it was found that the extracellular potential changes which were produced by the action current were different with respect to sequence duration and amplitude Thus impulses recorded at the soma and at the axon between the cell body and a point 200—300 μ distant from the axon hillock were triphasic indicating the flow of a net membrane current in an outward, inward, and outward direction at and near the recording electrode (Fig 1 and 8) The amplitudes of the potential changes were 2—4 mV if the electrode was placed close to the nerve cell membrane

Provided the cell was firing action potentials evoked by naturally occurring depolarizations, biphasic impulses reflecting an initial phase of inward, and later phase of outward membrane current were recorded at an axonal segment, some tens of microns in length which was located at a distance of about 200 μ from the axon hillock (Fig 5 and 8) The amplitudes of these potential changes were 2—3 mV When the cell was stimulated antidromically, the impulses became triphasic with an initial positive deflection (cf Edwards and Ottoson 1958)

Again, at the distal axon the impulses were triphasic, irrespective of the mode of stimulation However further distally than about 200 μ away from the above indicated axonal segment the potential changes could not be detected until the electrode was on the verge of impaling the axon their amplitudes did not exceed 0 5 mV Therefore, whenever it was necessary to register the propagation of action potentials along the axon this was done with a wire electrode placed against the axon in paraffin

In the dendritic region the impulses were biphasic the first positive deflection always being larger than the later negative one (Fig 1 and 8) Impulses of that kind could be recorded all over the dendritic region as far as 200—300 μ away from the dendritic bases At different points

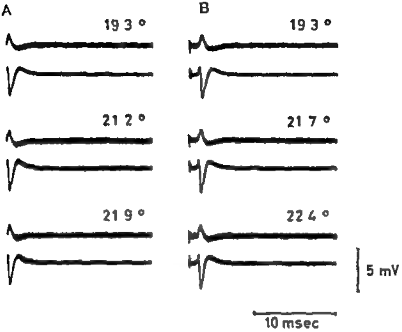
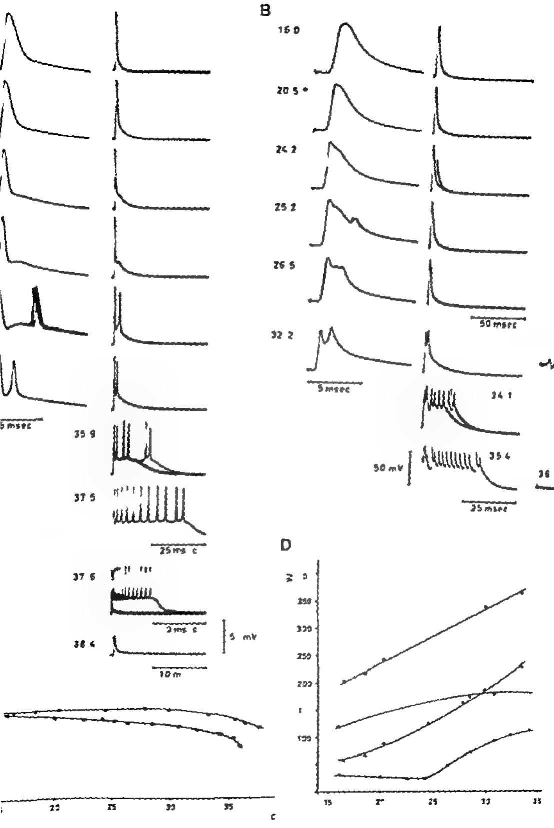


Fig 1 Simultaneous extradendritic (upper traces) and extrasomal (lower traces) recordings of discharges of a cell evoked at different temperatures by stretch of the receptor muscle A and by antidromic stimulation B Several traces are superimposed in each record The following points apply to this and subsequent illustrations (1) The temperatures at which the records were obtained appear on the frames (2) Upward deflections of the traces indicate positive going and downward deflections negative going potential changes (3) When recording *stretch evoked* discharges the oscilloscope sweep was triggered by the first action potential of each discharge

within this region the impulses had different heights but no distinct inverse relationship between this latter parameter and the distance from the cell body could be detected The maximal amplitude between the peak of the positive and the peak of the negative deflection was about 1 mV The transformation from the triphasic potential changes at the soma to the biphasic ones at the dendritic region was found to take place in a region at or near the dendritic bases (Fig 8)

By recording simultaneously at the soma and dendrites it was found that the outward flow of current across the dendritic membrane occurred at the same time as the initial outward and subsequent inward flow of current across the soma membrane the phase of inward current in the dendritic region coincided in time with the terminal phase of outward current at the soma (Fig 1) The latency between the peak of the inward current at the soma and that of the inward current in the dendritic region was about 0 75 msec at 20°C


B
16 0
20 5 *
24 2
25 2
26 5
32 2
50 msec
5 msec
34 1
50 mV
35 4
25 msec
35 9
37 5
37 6
38 4
5 mV
D
20
25
30
35
15
20
25
30
35
c

8

Intracellular recording Intrasomal recording was employed in most of the experiments Impalement of cell regions other than the soma was not often successful because the structures are small and surrounded by a tough connective tissue sheath Moreover, with the exception for their bases the dendrites are concealed by the tissue of the intertendon of the receptor muscle However in some cases it was possible to record intracellularly in regions other than the soma In two of these experiments, to be described here, the cells were impaled two times in succession each time at a different region in the third experiment to be described elsewhere (Grampp 1966b), recording was done simultaneously at two different sites

In the first experiment a cell was impaled for the first time, at a dendrite about 100 μ away from the dendritic bases and, for the second time, at the axon about 200 μ distant from the axon hillock The discharges which were recorded in these cell regions at different temperatures are shown in Fig 2 A and B The extracellular recordings were obtained when the electrode slipped out from the dendrite due to a slight dislocation of the preparation In the other experiment the cell was impaled for the first time at the axon at a distance of about 1200 μ from the axon hillock and, for the second time at the soma The discharges which were recorded in the indicated parts of the cell at various temperatures are shown in Fig 3 A and B All discharges were elicited by antidromic stimulation

The following findings were made in these experiments

(1) Full sized action potentials indicating an invasion by the active process of the membrane around the electrode were recorded not only in the axon and soma but also in the dendrites about 100 μ away from the

---

Fig 2 Recording at different regions of a cell at different temperatures A Discharges evoked by antidromic stimulation of the relaxed cell recorded intracellularly in the axon about 200 μ distant from the axon hillock Several traces are superimposed in the records at 30 0 33 5 33 9 37 6 and 38 4°C Time calibrations 5 msec for records in the left column and 25 msec for records in the right column unless indicated otherwise B Discharges evoked by antidromic stimulation of the relaxed cell recorded intracellularly in a dendrite about 100 μ away from the dendritic bases (all records except the two to the right) and extracellularly (the two records to the right) after the electrode had slipped out from the dendrite due to a slight dislocation of the preparation Several traces are superimposed in all records except at 35 4 and 36 0°C Time calibrations Intracellular records 5 msec for records in the left column and 50 msec for upper five and 25 msec for lower three records in the right column Extracellular records 5 msec for record at 32 2°C and 25 msec for record at 36 0°C The voltage calibration does not apply to the extracellular records a c amplification was used also for intracellular recording of the discharges shown in B C The variation with temperature of the amplitudes of the first action potential of the intraaxonally (filled circles) and intradendritically (open circles) recorded discharges shown in A and B respectively D The variation with temperature of the graphically derived maximal rate of rise (circles) and maximal rate of fall (triangles) of the first action potential of the intraaxonally (filled symbols) and intradendritically (open symbols) recorded discharges shown in A and B respectively

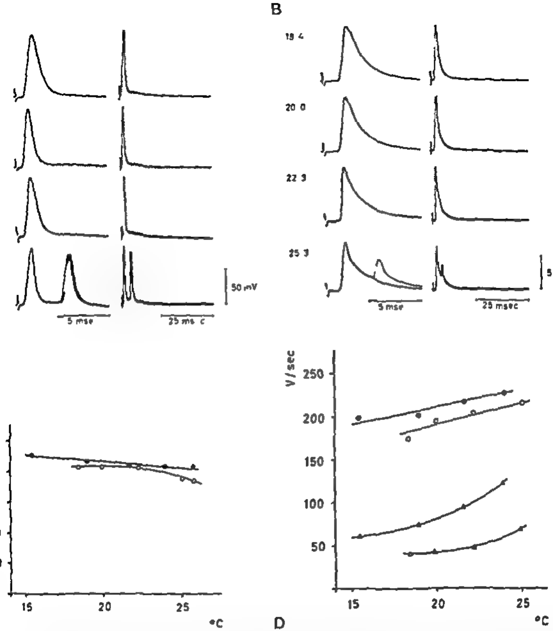


Fig 3 Recording at different regions of a cell at different temperatures A Discharges evoked by antidromic stimulation of the relaxed cell recorded intraaxonally at a distance of about 1200 μ from the axon hillock Several traces are superimposed in each record B Intrasomally recorded discharges evoked by antidromic stimulation of the relaxed cell Several traces are superimposed in each record C The variation with temperature of the amplitude of the first action potential of the intraaxonally (filled circles) and intrasomally (open circles) recorded discharges shown in A and B, respectively D The variation with temperature of the graphically derived maximal rate of rise (circles) and maximal rate of fall (triangles) of the first action potential of the intraaxonally (filled symbols) and intrasomally (open symbols) recorded discharges shown in A and B respectively

dendritic bases (see also Grampp 1966b Fig 6) The first impulse of those multiple-spike discharges that were registered after the electrode had slipped out from the dendrite were of a biphasic kind typical for the dendritic region of all cells studied.

(2) There were differences in time course between the action potentials of different cell regions Thus, at any temperature the slope of the depolarizing and preferably that of the initial part of the repolarizing phase was steeper (Fig 2 D and 3 D) and the degree of repolarization produced by the initial rapidly repolarizing phase before the onset of a final slowly repolarizing phase was greater for intraaxonally than for intrasomally or intradendritically recorded spike potentials. Besides this the peak of the former was more pointed than that of the latter spikes. The same differences, although less conspicuous were found to exist between soma action potentials, on one hand and dendrite action potentials on the other hand (Grampp 1966b Fig 6) The amplitude of intraaxonally recorded impulses was some millivolts higher than that of intrasomally or intradendritically recorded action potentials (Fig 2 C and 3 C)

The invasion of the dendritic membrane by the active process

In order to find out whether the negative deflection of extradendritically recorded impulses was caused by an active depolarization of the dendritic membrane near the recording electrode (Tasaki *et al* 1954 Chang 1955 Cragg and Hamlyn 1955 Fatt 1957 Andersen 1960 Fujita and Sakata 1962 Hild and Tasaki 1962) or by a depolarization due to electrotonic spread from the actively depolarized soma (Rall 1962, Nelson and Frank 1964) it was studied how the extrasomal and extradendritic potential changes produced by antidromically evoked discharges were modified during the action of xylocain which was applied to the dendritic region of the cell (Fig 4) A significant decrease of the amplitude of the negative deflection of the extradendritically recorded impulses and that of the terminal positive deflection of the extrasomally recorded impulses was seen already about 20 sec after the application of xylocain had been started. Later during the action of the anaesthetic these phases were almost abolished. At the same time there was considerably less, or no reduction in height of the negative deflection of the extrasomal, and of the simultaneous positive deflection of the extradendritic potential changes respectively From these findings it is inferred that the negative deflection of the extradendritically recorded impulses does represent an active depolarization of the membrane

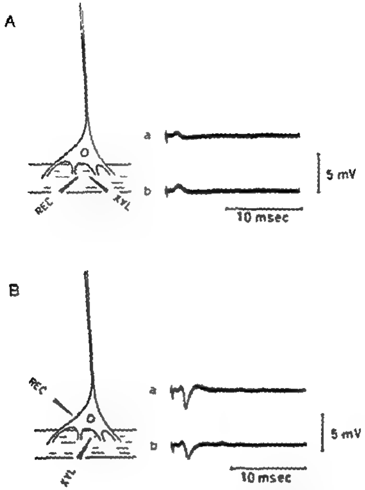


Fig. 4. Discharges of a cell evoked by antidromic stimulation which were recorded extradendritically (A) and extrasomally (B) before (a) and 20 sec after (b) an application of xylocain to the dendritic region had been started. Several traces are superimposed in each record. The anaesthetic was injected with a micropipette into the intertendon of the receptor muscle (see schemes to the left. REC: recording electrode; XYL: pipette for injection of xylocain). The experiment was performed at 16.5°C.

Firing with multiple spike discharges

Extracellular recording. It never occurred, no matter at what region of the cell they were recorded, that the impulses of a multiple spike discharge differed from each other with respect to the sequence of their phases (Fig. 8). This was particularly evident when the spike frequency within a multiple spike discharge was low. However, as it increased with rising temperature, a decrease of the amplitudes of one or several phases

of a number of impulses succeeding the first spike of the discharge was observed The number of impulses that were reduced and the degree of reduction was different at different parts of the cell

Thus even when fired at the highest naturally occurring frequencies there was little or no decrease of the negative phase of those impulses that were recorded at a particular axonal segment which, being some tens of microns in length was located within that part of the axon at which extracellularly recorded impulses were seen to start with a negative deflection (Fig 5 and 8) In addition the inward phase of the action current recorded at the indicated axonal segment always preceded in time that of the same impulse recorded at other parts of the cell (Fig 5) Such a behavior which is held to be typical for the region of origin of propagated action potentials (Edwards and Ottoson 1958) was never observed at other parts of the cell It is concluded therefore, (1) that the slowly adapting stretch receptor neuron of the lobster has only one spike-trigger zone which is located in the axonal segment in question and (2) that the membrane properties which are responsible for the triggering of propagated action

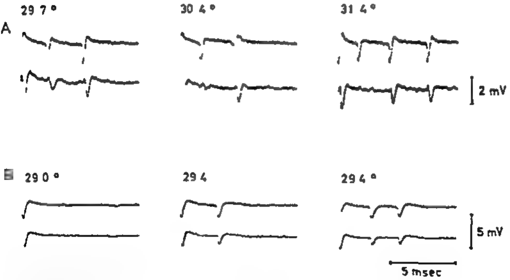
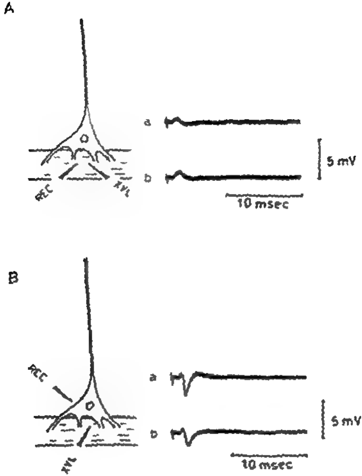


Fig 5 A Stretch-evoked multiple spike discharges of a cell at different temperatures recorded extracellularly and simultaneously at the axon about 200 μ distant from axon hillock (upper traces) and at the soma (lower traces) B Stretch-evoked discharges of another cell at different temperatures recorded extracellularly and simultaneously at the axon about 200 μ distant (upper traces) and 100 μ distant from the axon hillock (lower traces) The discharges at 29 4°C are multiple spike discharges



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and soma (Fig 5 A), and between the soma and dendritic region (Fig 8 26 0°C third impulse) respectively

The amplitudes of the extra impulse(s) of multiple spike discharges which were recorded at the distal axon usually were reduced only slightly even at high spike frequencies (Fig 7 A) However, sometimes the reduction of the impulse amplitude was significant (Fig 7 B) or a number of impulses often in the beginning of the multiple-spike discharge could become blocked completely between the spike-trigger zone and the recording site at the distal axon (Fig 8 30 7°C)

Intracellular recording The present findings were obtained in the two previously described experiments in each of which intracellular recording was made in two different parts of a cell (Fig 2 and 3) The amplitudes of the extra action potential(s) of intraaxonally recorded multiple-spike discharges with high spike frequencies were reduced much less than those of the extra impulse(s) of intrasomally or intradendritically recorded multiple spike discharges At high temperatures there was a slight increase in height of the extra spikes during the course of those multiple-spike discharges that were registered in the initial segment of the axon (Fig 2 A 35 9—37 6°C) The maximal rates of depolarization and repolarization were smaller for the extra impulse(s) than for the first action potential of multiple spike discharges with high impulse frequencies no matter in what part of the cell they were recorded However, the degree of decrease of these characteristics from the first to the following spike(s) was considerably smaller in axonal than in somal or dendritic multiple-spike discharges

The effect of temperature variations on the conduction of the active process along the membranes of different cell regions

At the same time as the temperature was varied in order to provoke firing with different discharge types the change of the average velocity of propagation either of single action potentials or of the extra impulses of multiple spike discharges was studied The rate of increase of the conduction velocity with rising temperature was the same for all types of impulses (which were not blocked) no matter along what parts of the cell they were travelling the Q_{10} was 1 6 (S D = ± 0 1) between 15 and 30°C In Fig 12 (triangles) is shown the effect of temperature on the time for conduction of action potentials along the axon between a point 3 7 mm distant from the axon hillock and the cell body The shorter values (open triangles) were obtained when the membrane was completely recovered

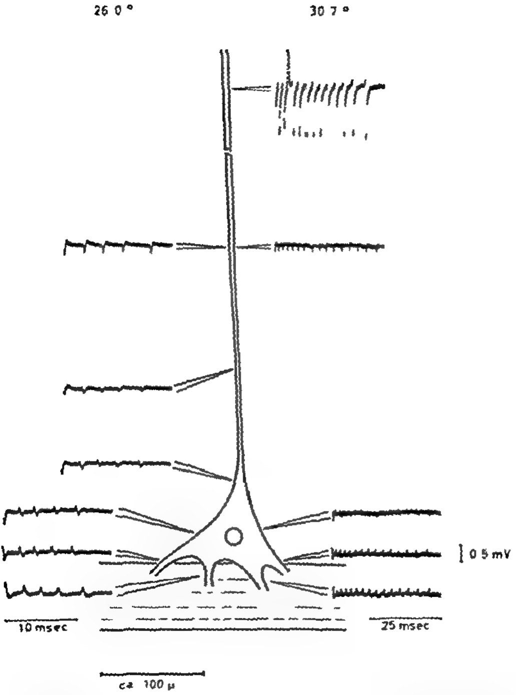


Fig. 8. Stretch e oke multiple spike discharges recorded extracellularly with a steel electrode at the d a ed reg ons of a cell (schematically drawn) at two different tempera u es. The ischarge from the distal end of the axon was recorded under paraffin

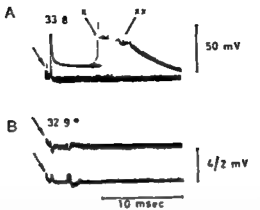


Fig 9 Simultaneous intrasomal (upper trace) and extradendritic (lower trace) recording A and extrasomal (upper trace) and extradendritic (lower trace) recording B of multiple spike discharges evoked by antidromic stimulation of a relaxed cell at temperatures at which the conduction of the first impulse of the discharges was blocked Several traces are superimposed in each record Voltage calibrations 50 mV for the intrasomal record 4 mV for the extrasomal record and 2 mV for the extradendritic records. Arrows point to stimulation artifacts ╲ indicates the initial part of the retouched rising phase of a full sized action potential Parts of traces showing extra spikes following the full sized action potential are seen at ╲╲

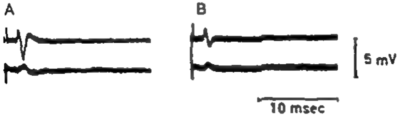


Fig 10 Simultaneous extrasomal (upper traces) and extradendritic (lower traces) recording of discharges of a cell evoked by antidromic stimulation at a low frequency A and at a high frequency B Several traces are superimposed in each record The experiment was performed at 25 0°C.

and the longer values (filled triangles) when it was refractory after a preceding active depolarization (Tasaki 1959)

Besides this expected behaviour it was found that in antidromically stimulated relaxed cells a decrease in height of intracellularly recorded action potentials (Fig 9 A and 11), or a reduction or abolition of the negative deflection of extracellularly recorded impulses (Fig 9 B) occurred when the temperature was raised above a critical value This value was different for different cells it became lower when the rate of antidromic stimulation was increased (Fig 10 and 11) Sometimes the decrease in

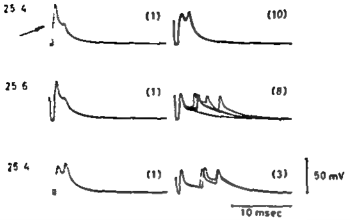


Fig 11 Intrasomal recording of discharges of a relaxed cell evoked at different temperatures by antidromic stimulation both at a low frequency (left column the numbers of stimuli per second are given in brackets) and at frequencies critical for provoking a block for conduction of action potentials (right column) The temperature of 26 4°C was critical for provoking a conduction block also at the low rate of stimulation Several traces are superimposed in each record Arrow indicates stimulation artifact

impulse height appeared suddenly (Fig 2 A *37 6°C)* but in the majority of cases it developed gradually, although at a rapid rate as the temperature and/or the stimulus frequency were approaching their respective critical values (Fig 2 B and C, *32 2—35 4°C)*

Because of taking place so abruptly, almost in an all or nothing fashion, the transformation from full sized to small impulses strongly indicates a blockage of the propagation of the active process (cf Grampp 1966b) This view is supported by the fact that at all stimulus frequencies the temperature-evoked block could be abolished again in an all or nothing fashion by slightly stretching the receptor muscle It was overcome also by antidromically evoked action potentials which invaded the cell body, while the latter was depolarized by an after depolarization which often followed a blocked impulse (Grampp 1966b) (Fig 12 inset, *19 0—22 0°C)* or by those action potentials that were evoked by the after depolarization itself (Fig 9)

In all cells investigated all phenomena related to the temperature-evoked blockage of the propagation of the active process could be reproduced an arbitrary number of times by raising and lowering the temperature and/or increasing or decreasing the frequency of antidromic stimulation

By recording extracellularly (and simultaneously) at different parts of the cell it was tried to find out in which membrane region the heat evoked

blockage of the impulse conduction was taking place It was seen that the negative deflection of the impulses was abolished between the soma and the dendritic region (Fig 9 and 10) It was noted, though, that this abolition was paralleled by an appreciable reduction of the amplitude of the negative phase of the soma impulses The significance of this latter reaction is not obvious Because of their invariable time course in successive discharges (see superimposed traces in Fig 9 and 10) the small soma impulses are not likely to reflect a local (or partial) activation of the somatic membrane It is not probable either that they are indicative of a blockage of the active process already in the initial segment of the axon or at or near the axon hillock, because this ought to result in a distinct decrease in height also of the positive deflection of the extradentritic potential changes In the present experiments such a decrease was either small or absent It is tentatively assumed therefore that the temperature-dependent blockage of the impulse conduction takes place first of all near the somato-dendritic border However, from the fact that the amplitude of intraaxonally recorded impulses was reduced abruptly when the temperature was raised above a critical value (Fig 2 A *37 6—38 4°C)* it is inferred that the propagation of the active depolarization may become blocked also in other parts of the cell

A particular behaviour which may reflect the fact that the membrane in different cell regions is able to fire at different maximal frequencies was sometimes observed in experiments of a type shown in Fig 12 The purpose of these experiments was to determine the effect of temperature on the least interval between two effective stimuli The stimuli were applied to the distal axon in the present case at a point 3 7 mm distant from the axon hillock and the effectiveness of the stimulation was checked by intrasomal recording (see inset the blockage of the conduction of action potentials which occurred at temperatures above 19 C is assumed to take place near the recording electrode and consequently to be of no importance when determining the reactivity of the axonal membrane) The result of the experiment is in accord with that of classic investigations (Gasser 1931 Tasaki and Fujita 1948) as far as the change of the duration of the functional absolutely refractory period with varying temperature (circles) is concerned However besides this it was found that at 17 0°C the minimal latency between two stimuli each eliciting an intrasomally recordable action potential was 7 80 msec instead of 2 65 msec which latter value was interpolated from the latency values which were determined at other temperatures As there is no reason to think that the length of the recovery cycle at 17 0°C was about the double of that at 15 0 C this fact can be explained only by assuming that the depolarization of the somato-dendritic membrane produced by the first antidromically evoked action potential initiated a second action potential in the spike trigger membrane from which place it propagated orthodromically along the axon and so long as it had not passed the stimulating electrode abolished by collision all spikes which travelled antidromically between the site of stimulation and the *spike trigger zone* Consequently a second impulse which was able to invade the cell body could be elicited not before the second action potential had passed the site of stimulation and the absolute refractoriness left behind there had subsided From the described *behaviour which is* believed to be a counterpart to the phenomenon of recurrent discharge which is seen in a fraction of antidromically stimulated motoneurons (Eccles 1955) *it is inferred that* the membrane of the spike trigger zone had a considerably shorter recovery cycle than that of the somato-dendritic region

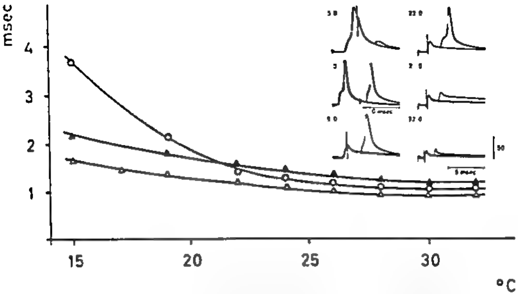


Fig 12 The effect of the temperature on the least interval between two effective stimuli applied to the distal axon of a cell (open circles) and on the time for conduction of action potentials along the fully recovered (open triangles) and the recovering (filled triangles) axon between the site of stimulation and the cell body The axon was stimulated at a point 3 7 mm distant from the axon hillock with supra threshold shocks of 0 1 msec duration The impulses evoked by this stimulation were recorded intrasomally (inset) Several traces are superimposed in the records each showing a pair of impulses which was evoked at the indicated temperature by two shocks the interval between which was critical for a 50 per cent effectiveness of the second stimulus The blocking of the conduction of action potentials which occurred at temperatures above 19 0 C is assumed to take place near the recording electrode and consequently to be of no importance when measuring the reactivity of the axonal membrane Note different time calibration for record at 17 0 C. — The least interval between two effective stimuli (open circles) was measured between the shock artifacts The time for conduction of the first (open triangles) and of the second action potential (filled triangles) of each pair of impulses was measured between the shock artifacts and the midpoints of the sharp deflections from the base lines" of the intrasomally recorded potential changes The value for the least interval between two successive stimuli each eliciting an intrasomally recordable response which was obtained at 17 0 C is not included in the curve for reasons described in the text The value for the conduction time for the action potential evoked by the second stimulus at 17 0 C was not determined

The effect of temperature variations on the time course of impulses recorded at different cell regions

Some observations concerning conspicuous changes in time course of impulses recorded extracellularly and intracellularly at different cell regions were made as the temperature was varied in order to provoke shifts from firing with one to firing with another type of discharge The following description applies only to potential changes that were produced by an active depolarization whose propagation was not blocked in the vicinity of the recording electrode

Extracellular recording With rising temperature there was a shortening in duration and a simultaneous increase in amplitude of all phases of the extracellular potential changes no matter at what part of the cell they were recorded (Fig 1)

Intracellular recording In all of a great number of experiments in which discharges were recorded intrasomally it was found that with rising temperature there was an increase of the maximal rates of depolarization and repolarization but a decrease of the amplitudes of the action potentials (Grampp unpublished results) The same held true for spikes which were recorded in cell regions other than the soma (Fig 2 and 3) However the rates at which the indicated parameters changed with varying temperature were different for action potentials recorded in different parts of the cell Thus the rates of depolarization and repolarization of soma or dendrite impulses increased more slowly with rising temperature than those of axon spikes while the amplitudes of the latter were reduced more slowly than those of the former action potentials (Fig 2 C and D and 3 C and D)

Actually, in the experiment shown in Fig 2 C the height of the intra axonally recorded action potentials increased slightly, while the temperature was raised through a certain interval In some other cases the amplitudes of intrasomally recorded spike potentials reacted in a similar way or remained constant during temperature variations within certain limits (cf Fig 3 C) However, a behaviour of that kind lasted only for the period which followed the impalement of the cell and an ultimate reduction of the impulse height never failed to appear provided the temperature was allowed to reach sufficiently high values It seems to be likely therefore that in these cases the spike amplitude changed not only with temperature but also with time i e it increased as possible lesions in the membrane around the impaling electrode became sealed

Stimulation by extracellular application of current to different cell regions

Since it was found that the conduction of the active process could become blocked in certain cell regions it was tried to estimate the excitability of the membrane in different parts of the cell This was done by stimulating the cell at different points with current applied with an extracellular microelectrode which had been placed close to the neuronal membrane (Fig 13) It was found that the polarity and minimal intensity of current (traces marked c) which was able to initiate spike potentials that could be recorded at the distal axon (traces marked b) were different at different

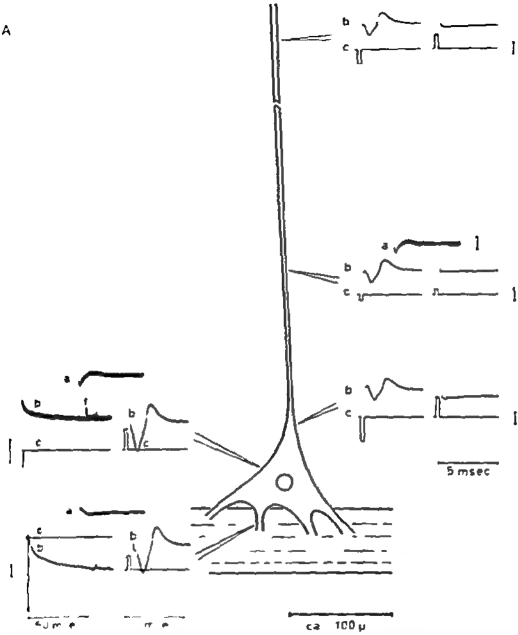
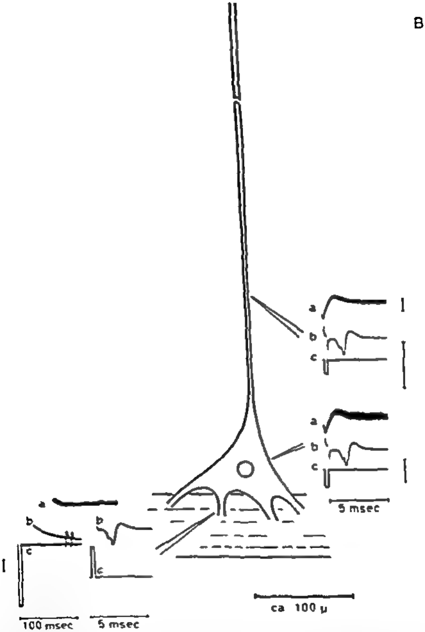


Fig 13 [illegible] Stimulation by application of current to different regions of a cell with an extracellular microelectrode A and B represent experiments performed on two different cells The traces marked a show the polarity and minimal amplitudes which square current pulses that were applied to the indicated regions of the cells (schematically drawn) must have in order to elicit action potentials which could be recorded extracellularly at the axon in paraffin at a distance of about 7 mm from the axon hillock (traces marked b) The duration was 0.3 msec for all current pulses but one which was applied to the dendrites of the cell shown in B and which lasted 6 msec. Traces marked b and a were recorded simultaneously Downward deflections of the traces marked b indicate positive going and upward deflections negative going potential changes Downward deflection of the traces marked c indicates cathodal, and upward deflection anodal current Vertical bars beside



current traces correspond to 2 μA — In order to eliminate as far as possible difference between the resistances due to structures interposed between the tip of the stimulating electrode and the neuronal membrane at the different sites of stimulation the current pulses were applied not until the amplitudes of stretch-evoked impulses which were recorded extracellularly with the stimulating electrode (traces marked a) indicated that the latter had been placed close to the nerve cell membrane The cells were relaxed before the eletrical stimulation was started The vertical bar beside the traces marked a at the initial segment of the axon corresponds to 1 mV the same voltage calibration applies to all other traces marked a Several traces are superimposed in all records The experiments were performed at 20 0 C.

reversal of its polarity were necessary when the stimulating electrode was moved along the axon near the axon hillock or the axon hillock itself and, also, along the cell region near the dendritic bases

The fact that in certain parts of the cell it was impossible to evoke propagated impulses by drawing current from the membrane underlying a small electrode, while the same membrane was able to produce full-sized action potentials when invaded antidromically, shows that local stimulation with a point source may be an unreliable test for electrical excitability (cf Grundfest 1961)

DISCUSSION

There is little doubt that those full sized action potentials and impulses with a negative deflection that could be registered intracellularly and extracellularly respectively, at the axon or soma of the present cell were signs of an active depolarization of the membrane at the recording site This seems to hold true for the corresponding dendritic impulses At least it is not likely that the latter were indicative of passive membrane potential changes in the dendritic region resulting from the spike invasion of the soma (cf Rall 1962 Nelson and Frank 1964) This is inferred from the following observations (1) On their way from the soma to the dendrites full sized action potentials decreased in height proportionately much less than impulses that obviously were caused by electrotonically conducted transients (cf Grampp 1966b, Fig 6) (2) Impulses of the latter type travelled from the soma to the dendrites at a higher speed than full sized spike potentials (or the corresponding extracellular potential changes) The average conduction velocity of the latter was 0 1—0 2 m/sec within the indicated cell region Similar values have been given for the speed of propagation of impulses, held to reflect an active process, in the dendritic region of a number of mammalian nerve cells (Chang 1955, Cragg and Hamlyn 1955 Fatt 1957 Hild and Tasaki 1962) (3) During the action of xylocain applied locally to the dendrites extracellular potential changes indicating the active depolarization of the latter decreases in amplitude more than those reflecting the spike activity in the soma It is thus concluded that in the slowly adapting stretch receptor neuron of the lobster the ability to depolarize actively is a property not only of the axonal or somatic but also of (parts of) the dendritic membrane

In the present case the antidromic invasion of the soma and dendrites by the active process may be essential for the generation of the slow regular rhythm of repetitive discharge activities evoked by continuous stretch of the receptor muscle or intracellular injection of a steady cathodal current This inference which concurs with a conception of Eccles (1953, 1964) about the functional significance of the spike activity in the somato--dendritic region of the motoneuron, is based on the following considera tions It is presumed that in the stretch receptor neuron the regenerative activity ends with a phase of active repolarization as it does in other nervous elements investigated (cf Hodgkin and Huxley 1952, Coombs

et al 1955 Julian *et al* 1962) If this holds true it may be possible that a depolarization such as the generator potential is eliminated in those parts of the cell that are invaded by the active process A consequence of this must be that in a repetitive activity driven by the generator potential the latter has to be reformed after each discharge in the dendrites and soma as well as in the spike trigger zone Because of the large area especially of the two former regions this process is likely to take longer time than the recovery from refractoriness in the spike trigger membrane This argumentation is consistent with the fact that each discharge in a repetitive activity of the above mentioned kind is succeeded by a phase of rapid repolarization taking place initially during the course of the so called after hyperpolarization[1] and a phase of slow depolarization which ends with the initiation of the next discharge This course of potential changes was found to be essentially independent of whether the repetitive activity was evoked by the cell s own generator current or by a steady *intrasomally* injected cathodal current (see Grampp 1966a Fig 4) Hence short circuiting of the generator current by the active process and/or direct interference between the latter and the generator current producing mechanism (cf Eyzaguirre and Kuffler 1955b) cannot play an exclusive role in the determination of the slow rhythm of stretch evoked repetitive activities Nor does this rhythm seem to be controlled by refractoriness and accommodation (cf Fuortes 1957 Fuortes and Mantegazzini 1962) This conclusion is based on the fact that during the course of prolonged depolarizations (after depolarizations) such as were seen to occur in multiple spike discharges (cf Grampp 1966a c) the spike-trigger membrane was able to fire for hundreds of milliseconds at frequencies which were manifold higher than those of repetitive activities

In the stretch receptor neuron there seems to be a regional differentiation not only with respect to the ability to produce a depolarizing current (generator current) (Eyzaguirre and Kuffler 1955a) or a repolarizing current (in response to an activation of the inhibitory system) (Kuffler and Eyzaguirre 1955) but also with respect to the degree of depolarization necessary for initiation of the regenerative activity and the time course of the latter As far as the firing level is concerned a regional differentiation is evident from the fact that, although the amplitude of the generator potential decreases in a direction from the dendrites towards the distal axon the propagation of the stretch evoked impulses always

[1] During the course of the after hyperpolarization the membrane became hyperpolarized not in relation to the resting level but as has been pointed out by Eyzaguirre and Kuffler (1955b) in relation to the potential level of the steadily depolarized cell

started in the spike trigger zone, a short axonal segment located about 200 μ distant from the cell body Hence, the threshold for impulse initiation must rise in a direction from this axonal segment towards the dendrites The same conclusion is drawn from the results obtained when estimating the excitability of different cell regions by local application of current pulses with an extracellular microelectrode Usually, the excitability changed markedly along the axon hillock and the dendritic bases respectively This means that the above mentioned rise of the threshold for impulse initiation is likely to be a discontinuous one The first step occurs between the spike trigger zone and the initial segment of the axon the second step at the axo somatic and the third step at the somato dendritic border zone Since the dendritic membrane did not respond to stimulation with a point source, nothing can be said about possible changes of the firing level farther out in the dendritic region (cf Grampp 1966b) The results obtained when estimating the excitability of different cell regions also indicate that the threshold for impulse initiation is likely to be somewhat higher in the distal axon than in the spike trigger zone

The following observations were made when estimating the excitability of different cell regions by extracellular application of current pulses (1) Changes in excitability were noted along regions of constant cellular dimensions (2) Although most likely they represented a smaller load than the soma the dendritic stems always were less excitable than the latter cell region From this is concluded that the differences in weight of the load presented to the electrode at the various sites of current application are not so large as to render the present technique unsuitable for a determination of possible variations in excitability between different membrane regions

The rise of the threshold for impulse initiation, but also the increase in membrane area at the axo somatic, and somato dendritic border respectively must imply a decrease of the safety factor for impulse propagation in each of the mentioned cell regions This conclusion is consistent with the finding that the conduction of impulses was blocked in either of these particular regions whenever a general reduction of the safety factor was produced by raising the temperature (Presumably this general reduction of the safety factor is due to a decrease of the membrane resistance and an increase of the membrane potential the firing level being constant Grampp unpublished results) There is some evidence that in the cases investigated the impulses were blocked in the first place at or near the dendritic bases and only in the second place after further raising of the temperature at or near the axon hillock From this is concluded that the safety factor decreases in a direction from the soma to the dendrites and that this decrease is a discontinuous one

The regional differentiation with respect to the rate of the regenerative

activity is evident both from the differences in time course of action potentials recorded intracellularly in various parts of the cell and from the differences in the degree of reduction in height of the extra spikes of multiple-spike discharges with high impulse frequencies Judging from these criteria the present finding indicate that while the active process travels from the spike trigger zone towards the dendrites its rate and hence, that of the recovery decreases in steps These steps occur at the axo somatic and somato dendritic border, respectively In the distal axon the rate of the regenerative activity is likely to be somewhat slower than in the spike-trigger zone

Thus there seems to be a regional correlation between the height of the threshold for initiation of the regenerative activity and the rate of the latter From this it may be inferred that both parameters are dependent on identical membrane characteristics In all cases investigated it was seen that the most conspicuous changes of the parameters in question occurred in approximately the same usually quite well defined cell regions i e on either side of the spike-trigger zone at the axon hillock and at the dendritic bases Thus, with respect to functional properties the slowly adapting stretch receptor neuron of the lobster can be subdivided into the distal axon, the spike-trigger zone the initial segment of the axon the soma and the dendritic region

The regional differentiation of the stretch receptor neuron is similar to that of the motoneuron Evidence has been given that in the latter cell steep rises of the firing level occur at the axo somatic (Coombs *et al* 1957a, b Fuortes *et al* 1957) and probably also at the somato dendritic (Fatt 1957) border zone Besides this there is a successive increase of the duration of the refractory period in a direction from the axon towards the dendrites (Brock *et al* 1953) A regional differentiation with respect to the characteristics of the active depolarization has also been noted in other types of cells Thus the giant neuron of *Aplysia* is subdivided into several regions which in a direction from the spike trigger zone towards the soma are increasingly less excitable (Tauc 1962a, b)

In an attempt to form a conception of the physical background of the observed regional differentiation of the membrane of the stretch receptor neuron it is assumed following in that respect a line of thought set forth by several authors (e g Fuortes *et al* 1957, Loewenstein 1961) that the specific ionic permeability changes do not involve the whole membrane but merely sites, or current generating units (perhaps of molecular dimen sions cf Eccles 1960) which are fitted into a membrane framework having the electrical properties of a leaky cable This is not unconceivable because

provided the ions are allowed to travel freely along their electro chemical gradients, only a minor fraction of the total membrane area is needed for passage of the ionic fluxes that give rise to a full sized action potential. It is also assumed that the different currents, i e the action current, the depolarizing current (generator current), and the re (hyper-)polarizing current (inhibiting current) are generated by different types of current generating units which are supposed to function principally independently of each others activity

On the basis of these premises the regional differences of the functional properties which became apparent in the present investigation can be explained just by assuming that the number per unit membrane area of the different types of current generating units is different in the various regions of the cell membrane. Thus the ability of the dendritic membrane to produce both action current, generator current and since there are inhibitory synapses repolarizing current is thought to be due to the simultaneous occurrence of all three types of current generating units. The generator current producing units are probably missing in the soma and in the axon there are likely to be only action current generating units. The approximate distribution of the latter over the cell membrane can be derived from the computation that a reduction of the values for the sodium and potassium permeability constants (P_{Na} and P_K) implies a rise of the threshold for impulse initiation and a decrease of the amplitude and the rates of rise and fall of the action potential (Frankenhaeuser and Huxley 1964). Provided the sodium and potassium permeability constants are mathematical expressions of the density per unit membrane area of the action current generating units, the findings of the present investigation indicate that there is a falling gradient of this density in a direction from the spike trigger zone towards the dendrites (Fig 14). Presumably this gradient is a discontinuous one, the discontinuities being between the spike trigger zone and the initial segment of the axon at the axon hillock and at the dendritic bases. In the distal axon the number per unit membrane area of action current generating units is likely to be somewhat less than in the spike-trigger zone. The different loading of the action current generators may be the cause of the regional differences in response to varying temperature of the time course of intracellularly recorded action potentials

It is appreciated that other explanations of the regional differentiation may be thought of. However the present one is attractive not only because it accounts well for the experimental findings described above and for a particular kind of after depolarization to be described elsewhere (Grampp

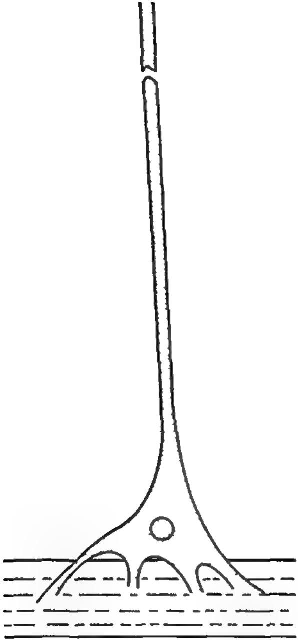


Fig 14 Hypothetical scheme showing the distribution of action current generating units (represented by dots) over the membrane of the slowly adapting nerve cell of the crustacean stretch receptor organ (For further details see text)

1966a), but also because it allows to regard regional differentiation as a matter of quantitative rather than qualitative variations of the membrane characteristics In this context it is interesting to note that evidence has been presented of a micellar structure of (parts of) biological membranes (Green and Fleischer 1964 Lucy and Dingle 1964) The possibility has also been considered that within a given membrane region there might exist side by side, different types, or arrangements of micelles each of which might be responsible for a particular membrane activity (Svennerholm) Following this line of thought it is not difficult to assume differences between different parts of a membrane with respect to the number per unit membrane area of the various kinds of micelles

SUMMARY

Firing with single spike and multiple spike discharges was registered with extra- and intracellular microelectrodes at different regions of the slowly adapting stretch receptor neuron of the lobster. At low spike frequencies extracellular impulses with a negative deflection and full sized intracellular spikes were recorded at the axon, soma, and dendrites. During application of xylocain to the dendritic region the reduction of the negative deflection was much greater for extradendritically than for extrasomally recorded impulses. This indicates that the ability to depolarize actively is a property not only of the axonal or somatic but also of (parts of) the dendritic membrane. The antidromic invasion of the soma and dendrites by the active process seems to be essential for the generation of the slow regular rhythm of repetitive activities evoked by stretch of the receptor muscle or by intracellular injection of a steady cathodal current.

In multiple spike discharges with high spike frequencies a number of impulses succeeding the first one were reduced in height. This reduction was least conspicuous or even absent at the spike trigger zone, an axonal segment some tens of microns in length, located about 200 μ distant from the axon hillock. There was, however, an increase of the degree of the reduction in a direction from the spike trigger zone towards the dendrites. Extracellular recording of multiple-spike discharges with very high spike frequencies showed that some of the extra impulses lost their negative (and subsequent) phases at the somato dendritic, or already at the axo somatic border. In intracellular recordings it was seen that a decrease in height of the extra impulses was parallelled by a reduction of their rates of rise and fall. From these findings it is inferred that there is a (stepwise) decrease of the rate of the active process, and hence of the subsequent recovery, in a direction from the spike trigger zone towards the dendrites. This idea is also consistent with the fact that the time course was faster for intraaxonally than for intrasomally or intradendritically recorded (extra) spikes.

Propagated action potentials were elicited by application of current pulses to different cell regions with an extracellular microelectrode. Cathodal current was found to excite the axonal membrane of all and the somatic membrane of most cells, whereas it was ineffective when applied to the dendrites of all and the soma of some cells. Application of anodal

current pulses to the electrically inexcitable cell regions resulted in the initiation of propagated action potentials presumably in or near the spike-trigger zone The electrically excitable and electrically inexcitable membrane regions were found to border on each other at or near the axon hillock in a few and at, or near the dendritic bases in most cells In the latter cases the minimal amplitude of cathodal current pulses that were able to evoke propagated action potentials had to be increased substantially when the stimulating electrode was moved along the axon hillock in a direction from the axon to the soma From these findings it is concluded that there is a rise (in steps) of the threshold for impulse initiation in a direction from the spike-trigger zone towards the dendrites This conception is also supported by the fact that the propagation of the active process could become blocked, presumably in the first place at the somato-dendritic border when the temperature was raised above a critical value

The regional differentiation with respect to threshold and rate of the regenerative activity as well as the fact that the characteristics of action potentials recorded in different cell regions changed at different rates with varying temperature is explained by assuming an uneven distribution over the membrane of the stretch receptor neuron of structurally fixed action current generating units

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in
a structural protein, resilin

BY

SVEND OLAV ANDERSEN

COPENHAGEN
1966

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36 Juliane Maries Vej Copenhagen Ø Denmark

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København, den 21. september 1965.

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Abbreviations

Contents

1 The diversity of covalent cross-links in proteins

The properties of a protein depend both on the linear arrangement of amino acid residues in peptide chains and on the spatial conformation of these chains. The conformation can be stabilized by non-covalent interactions as well as by covalent linkages: cross-links. By means of such cross-links several peptide chains can be interconnected or distant parts of a single chain can be joined together resulting in a loop.

Nearly all the covalent cross-links described so far occur only in extra-cellular proteins. An exception is the disulphide group (–SS–) which occurs both intra- and extra-cellularly, although it appears to be most common in extra-cellular proteins (*Cecil* 1963).

Several types of covalent cross-links have come to light only recently, and since the so-called stroma proteins of cells have been very little investigated so far, the possibility exists that they contain some hitherto unrecognized cross-links. On the other hand, stroma proteins from mitochondria and other membrane systems appear to be stabilized and rendered insoluble by very strong hydrophobic interactions (*Criddle et al.* 1962, *Richardson, Hultin* and *Green* 1963).

Covalent cross-links can provide a more stable connection between protein chains than non-covalent interactions, and their presence in extra-cellular structures may be of adaptive value. Different mechanisms of cross-linking are found in proteins, and a connection may be assumed between the chemical and physical properties of the structural proteins and the type of cross-link they contain (see chapter 8).

KERATINS

This group of proteins is present in hair, nail, feather and the epidermis in vertebrates. Keratins are formed and retained by cells which gradually become completely keratinized. Strictly speaking, it is therefore an intra-cellular protein. Keratin is stable, being rich in –SS– groups, and can only be dissolved after breakage of the sulphur bridges by reduction or oxidation.

COLLAGEN

Collagen is the most ubiquitous extra cellular protein, and it has been demonstrated in nearly all species (*Gross* 1963) A fraction of the collagen can be extracted by neutral salt solutions and labelled amino acids are incorporated faster into it than into the insoluble fraction The soluble fraction has a rapid turnover whereas the insoluble fraction has almost no turn over in most tissues and the soluble fraction is assumed to be a precursor of the insoluble fraction (*Harkness et al* 1954 *Orekovitch et al* 1960) As an animal gets older less of the collagen is soluble, this is believed to be due to a slow formation of cross links between the peptide chains an idea supported by finding that the three peptide chains involved in the collagen triple helix are easily separated in recently synthesized collagen, whereas this separation is not possible in older material (*Piez et al* 1961, *Grassmann et al* 1963) Moreover from enzymatic digests of collagen some peptides have been isolated which seem to consist of three interconnected chains (*Grassmann et al* 1960)

The nature of the cross links is unknown but several types have been proposed From partial digests of collagen *Mechanic* and *Levy* (1959) have isolated a small peptide with glutamic acid linked to the ε amino group of a lysine residue by means of peptide linkage The number of lysine residues involved in this type of cross linking is not known, as the number of free ε amino groups varies with the analytical method used Only 60–70 per cent of the ε amino groups in collagen reacts with fluorodinitrobenzene (*Bowes* and *Moss* 1953 *Solomons* and *Irving* 1958) whereas they can be quantitatively acetylated both in collagen (*Green et al* 1953), and in gelatine (*Kenchington* 1958) More than 90 per cent of the groups can be guanidated (*Betheil* and *Gallop* 1960) These differences may be due to steric hindrance preventing some of the reagents from getting access to all free amino groups

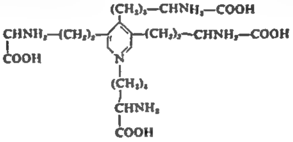
In addition collagen contains bonds which behave like ester bonds in being easily split by hydroxylamine and hydrazine during this reaction the molecular weight of the collagen is lowered (*Gallop et al* 1959) α and β carboxylic groups of aspartic acid residues are involved in the ester like bonds whereas the alcoholic groups involved have not been identified (*Blumenfeld* and *Gallop* 1962) It has been suggested that carbohydrates could function as cross links in collagen by being ester linked to two peptide chains (*Hörmann* 1960) However, in fish collagen (ichthyocol) the carbohydrates are linked to the peptide chains only by means of a single bond (*Blumenfeld et al* 1963)

Phosphate groups have been suggested to function as cross links in dentine

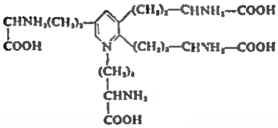
collagen (*Lees* and *Schlueter* 1963) by forming diesters with hydroxylic groups in serine and threonine residues The possible function of ester or amide linked phosphate groups as cross links in protein was discussed by *Perlman* (1955)

ELASTIN

Two unusual isomeric amino acids desmosine (A) and isodesmosine (B) have recently been isolated from hydrolysates of the elastic protein elastin (*Partridge Elsden* and *Thomas* 1963 *Thomas Elsden* and *Partridge* 1963) Both contain a pyridinium nucleus with four aliphatic sidechains with terminal carboxylic and α amino groups



A



B

It is possible that each of them functions as a cross-link between as many as four peptide chains Both compounds are apparently formed from protein bound lysine residues (*Miller Martin* and *Piez* 1964 *Partridge et al* 1964) supporting the idea that they are cross-links

BACTERIAL CELL WALLS

Diaminopimelic acid (C) has been isolated from hydrolysates of cell walls of several bacteria Together with lysine it seems to take part in the formation of cross links in the mucopeptides present in the cell walls The evidence

is that in some of the residues both amino groups are protected from reaction with fluorodinitrobenzene. These problems have recently been reviewed by *Perkins* (1963).

C

$$\begin{array}{c} COOH \\ | \\ CHNH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CHNH_2 \\ | \\ COOH \end{array}$$

SCLEROTIZED CUTICLE

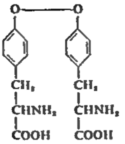
Another type of cross-linking is present in the sclerotized exocuticle in insects. Here a preformed protein becomes tanned after ecdysis apparently by means of a low molecular quinone formed by oxidation of a diphenolic compound (*Pryor* 1940a, b). The quinone reacts with the free amino groups present, such as amino endgroups and ε-amino groups in lysine residues. Since one molecule of quinone can react with more than one amino group this can result in cross-linking of the protein chains. The tanning of insect cuticle will be treated in more detail in chapter 8.

MOLLUSCAN SCLEROPROTEINS

Stegemann (1961, 1963) has shown that several unidentified phenolic compounds are present in hydrolysates of molluscan scleroproteins. These phenols are strongly bound to the proteins and he suggests that they could be responsible for the cross-linking. At any rate, quinone tanning is thought to be involved in such structures as the periostracum and the byssus threads in *Mytilus* (*Brown* 1952) and in the hinge ligament in *Pecten* (*Trueman* 1953a, b).

BARLEY PROTEINS

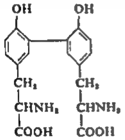
Davies and *Harris* (1961) have demonstrated that certain proteins from barley and malt contain a peroxide formed from two residues of tyrosine (D). The formation of this type of cross-link could be responsible for the insolubilization of some of the proteins during malt production.



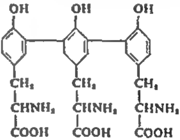
D

RESILIN

A new type of cross link found in the elastic protein from insects resilin can now be added to the list. Resilin contains two fluorescent amino acids (*Andersen* 1963) which recently have been identified as dityrosine (E) and trityrosine (F) (*Andersen* 1964). The presence of these amino acids in proteins has not been described before



E



F

The purpose of this paper is to give a detailed report of the identification of the two amino acids and to discuss the evidence for their function as cross links between peptide chains. A hypothesis for their biosynthesis is presented and the formation of resilin is compared with that of other cross linked proteins

2 Occurrence and general properties of resilin

OCCURRENCE

Resilin occurs in several cuticular structures of epidermal origin in arthropods (*Andersen* and *Weis-Fogh* 1964). It has been found in all insects investigated and also in a crustacean, the crayfish (*Astacus fluviatilis*) but appears to be absent from arachnids. Other groups of animals have not been investigated and it is possible that it is more widespread than indicated by these examples.

In the desert locust (*Schistocerca gregaria* Forskål), the animal we have used most, resilin is present in many structures, a list of which is found in a recent review (*Andersen* and *Weis-Fogh* 1964). In addition it has now been found in the pads of the tarsi (*Andersen*, unpublished), the identification being based on the autofluorescence of the structures and on the presence of the two fluorescent amino acids.

Most of the resilin-containing structures are either very small or resilin occurs in such close contact with other cuticular components that it is impossible to obtain pure samples. So far, only two structures from the locust and one from dragonflies (*Aeschna grandis*, *Aeschna cyanea*) have proved useful in our investigations (*Weis-Fogh* 1960). The dragonfly tendon is shown in Fig. 1; it has been photographed both in ordinary light and by its own fluorescence.

The tendon has form as a hollow airfilled tube which in the large *Aeschna* species is about 0.8 mm long and 0.15 mm in diameter in the swollen state at pH 7. It contains about 6 µg of dry matter (*Weis-Fogh* 1961 a). It is well suited for elastic measurements, since threads for pulling can easily be tied to both ends (*Weis-Fogh* 1961 a). These ends contain chitin and non-elastic proteins in contrast to the swollen elastic part which consists of pure resilin covered by thin membranes on both outer and inner surfaces (*Weis-Fogh* 1960). Due to the absence of chitin from the elastic part, the tendon is also well suited for studies of the swelling behaviour in different media, but the small size makes it awkward for chemical work. It has been possible to show

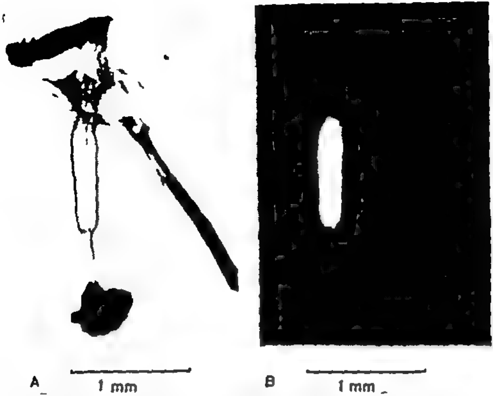


Fig. 1 Mesothoracic tendons from *Aeschna grandis*. After removal of the epidermal cell layer the tendons were placed in distilled water and photographed in ordinary light (A) and in the fluorescence microscope showing the autofluorescence (B). The swollen fluorescent part of the tendon consists of resilin whereas the non rubbery non fluorescent darkly coloured tendon is fully sclerotized.

that the two characteristic amino acids are present but, for the moment, it would be very difficult to determine the amounts accurately.

The two ligaments from the locust contain enough material for chemical work, but their form and structure make determinations of the physical properties complicated (*Jensen* and *Weis-Fogh* 1962). The ligaments are essential parts of the locust flight system and have been called the *wing hinge* and the *prealar arm* respectively. Figs. 2 and 3 show sections of the two ligaments photographed by their own fluorescence. In the prealar arm a banding can be seen which is due to a daily rhythm of deposition (*Neville* 1963 a).

The prealar arm is built up of alternating layers of resilin and chitin (Fig. 4), the thickness of the resilin layers being 1–3 μ and that of the chitin lamellae about 0.2 μ (*Weis-Fogh* 1960). The main part of the wing hinge has a similar structure, but it also contains a pad consisting of pure resilin (*Weis-Fogh* 1960) which is the last part of the ligament to be deposited

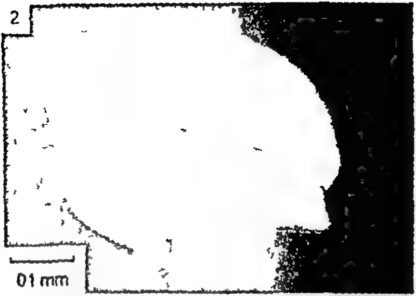


Fig 2 Section of a prealar arm from *Schistocerca gregaria* The arm was frozen sectioned (10 μ) without fixation Photographed in the fluorescence microscope by its autofluorescence Notice the differences in fluorescence corresponding to daily growth layers

Fig 3 Transverse section of wing hinge from the forewing of *Schistocerca gregaria* The hinge was frozen sectioned (10 μ) without fixation Photographed in the fluorescence microscope by its autofluorescence Notice the differences in fluorescence in the different parts The sclerotized cuticle (lower part of the figure) appears weakly fluorescent but it does not fluoresce with the blue colour characteristic for resilin

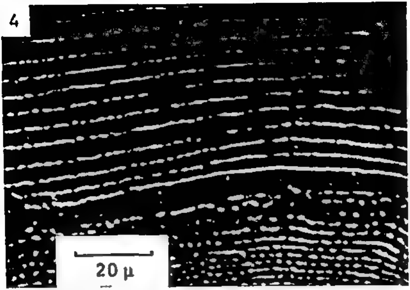


Fig. 4. Part of section (6 µ) of prealar arm from *Schistocerca gregaria* photographed in ordinary light to show the alternating layers of chitin (thin lamellae) and resilin (thick layers)

(*Neville* 1963 b). The presence of parallel chitin lamellae has the effect that a piece cut out from such a part of a ligament can only swell in one direction: normal to the lamellae.

The ligaments can be obtained by careful dissection under the microscope and manual removal of all adhering tissue and non-elastic cuticle. From one locust two wing hinges and two prealar arms can be obtained, amounting to about 0.3 mg material of which approximately 80 per cent is resilin and the remaining part is chitin (*Bailey* and *Weis-Fogh* 1961, *Neville* 1963 b). If the preparation of the wing hinges is not performed carefully, a second protein will be present which is distinct from resilin as it appears fibrous, non-elastic, strongly birefringent (*Weis-Fogh* 1960) and without fluorescence.

PROPERTIES

The two most outstanding properties of resilin are the elasticity and the insolubility. It is insoluble not only in water at temperatures below 140° C but also in all other solvents we have tried, provided that they do not rupture peptide bonds (*Weis-Fogh* 1960, *Andersen* and *Weis-Fogh* 1964). In many solvents resilin swells considerably, especially in those which are known as effective protein solvents (phenol, formamide, formic acid, etc.). It also swells

without going into solution in concentrated solutions of lithium thiocyanate and cupric ethylenediamine solvents which are able to dissolve silk fibroins and cellulose. If after the swelling has reached its maximum and no further changes can be observed in the samples, they are then transferred back to distilled water they return to their former size and shape. If the resilin samples are placed in absolute methanol, ethanol, dioxane or acetone they shrink and become hard and glass-like as when dried in air. If again placed in water, the samples swell and regain their rubbery elasticity and no differences can be noted between samples treated in this way and the fresh samples (*Weis-Fogh* 1960).

The swelling in water is pH-dependent (*Weis-Fogh* 1960, *Andersen* and *Weis-Fogh* 1964). It is least pronounced about pH 4, indicating that this is the isoelectric point of the protein. The swelling increases at lower and especially at higher pH values. This behaviour agrees with the amino acid content

Table I

Amino acid composition of resilin (averaged from *Bailey* and *Weis-Fogh* 1961 and from Table III in this paper)

	Resilin from locust ligaments	
	residues per 10^5 g protein	residues per 100 residues
Glycine	437	37.9
Alanine	126	10.9
Valine	32	2.8
Leucine	26	2.3
Isoleucine	19	1.6
Proline	88	7.6
Phenylalanine	29	2.5
Methionine	0	0
Serine	91	7.9
Threonine	35	3.0
Hydroxyproline	0	0
Tyrosine	31	2.7
Aspartic acid + asparagine	116	10.1
Glutamic acid + glutamine	53	4.6
Lysine	6	0.5
Histidine	10	0.9
Arginine	40	3.5
Dityrosine (Compound II)	9	0.8
Trityrosine (Compound I)	4	0.3
Tetratyrosine (Compound III)	trace	
Total	1152	99.9
Average residue weight	86.8	

(Table I) since resilin is rich in amino acids carrying charged groups nearly twice as many free acidic groups as basic groups being present (*Bailey* and *Weis Fogh* 1961) This should give a relatively low isoelectric point

The strong swelling in dilute alkali formamide and phenol indicates that resilin has a tendency to dissolve in these solvents but this must be counter acted by some factors keeping the chains together Covalent cross links be tween the peptide chains could explain this behaviour

Another characteristic property of resilin is its elasticity (*Weis Fogh* 1960) The elastic tendon from dragonflies can be stretched almost to three times its unstrained length without breaking and it returns immediately to its original length when the strain is released The unstrained tendon is com pletely isotropic but becomes positively birefringent in the direction of ex tension when strained No flow of material is observed during stretching and no lasting deformations are present even after the sample has been kept in the stretched condition for weeks on end *Weis Fogh* (1961 a b) has per formed a detailed analysis of the elasticity of dragonfly tendons and he came to the conclusion that resilin exhibits almost ideal rubberlike elasticity The force applied during the stretching has only little influence on the internal energy, the main effect being a decrease in the entropy of the material

Determinations of the extension and compression of resilin (*Weis Fogh* 1961 b) showed that the material corresponds to a model for a short-chain vulcanized rubber suggested by *Treloar* (1954) This model presupposes that four flexible half chains meet at each junction point that the chains are of approximately equal length and that they contain a relatively low number (5–25) of random links This model was found to correspond well with the experimental results regardless of the degree of swelling From the elastic modulus of tendon resilin it was calculated that the average molecular weight of the chains between two junction points is 5100 and later the corresponding value for locust resilin was calculated to about 3400 (*Jensen* and *Weis Fogh* 1962 *Andersen* and *Weis Fogh* 1964)

From the studies on the elasticity of resilin it was concluded that a stable secondary structure is absent when the protein is in the swollen state Be tween the junction points the chains must be free to take up a random con figuration and both inter and intra-chain hydrogen bonding must be nearly completely suppressed (*Weis Fogh* 1961 b)

Both the swelling properties and the elasticity thus indicate that stable links are present between adjacent protein chains keeping them in a given equili brium position relatively to each other This position can be influenced by changing the external conditions such as the composition of the medium or the forces applied The previous treatment of the sample would be expected

only to have a lasting effect if it brought about irreversible damage, such as breakage of peptide bonds or modification of side chain groups

The term junction points as used here does not only include covalent cross links of all possible types but also physical entanglements between the chains Entanglements alone cannot explain the behaviour of resilin, since this would not prevent the chains from sliding relatively to each other during stretching resulting in lasting deformations but physical entanglements may well be present in addition to some sort of stable linkages Evidence to be presented later (p 55) indicates that entanglements play only a minor role

If the polypeptide chains consist of large rings woven together in a huge network this would of course correspond to an effective cross linking, with out covalent linkages between the chains However it is highly improbable that two ends of a randomly coiled and thermally agitated polypeptide chain could meet after one of them has passed through a ring already formed (*Weis Fogh* 1961 b) Furthermore the presence of free amino endgroups in the protein indicates that the polypeptide chains are not rings

Another possibility is that the chains are held together at certain points by strong secondary forces while the rest of the chains are free to take up a random configuration These forces must be very strong and of an unusual type to resist the tendency of the chains to dissolve, and to be unaffected by changes in pH in the range from 1 8 to 12 3 (*Weis Fogh* 1961 b) That small crystalline regions keep the chains together can be excluded as X ray dif fraction studies have shown that no true crystallinity is present even in ten dons which have been dried slowly after being stretched almost to the break ing point (*Elliot Huxley* and *Weis Fogh* 1965)

The most plausible explanation must therefore be that stable covalent cross links are present connecting the peptide chains to a huge three dimen sional network The cross links cannot belong to any of the hitherto recognized types mentioned in the introduction Disulphide linkages can be excluded because amino acid analysis shows that resilin does not contain sulphur containing amino acids Resilin is completely stable towards reagents which break –SS–linkages either by reduction (alkaline thioglycolate) or by oxida tion (performic acid) (*Weis Fogh* 1960) Phosphate mediated cross links can not be present in resilin since phosphate determinations were completely nega tive and ester groups can also be excluded, because resilin is unaffected by alkaline hydroxylamine and by hydrazine (*Andersen* unpublished)

Dinitrophenylation of resilin was performed according to *Fraenkel Conrat Harris* and *Levy* (1955) and showed that 6–7 ε amino groups per 10^5 g resilin are free to react corresponding to the total amount of lysine in resilin (6 residues per 10^5 g resilin) About 7 amino groups per 10^5 resilin react with

ninhydrin including ε amino groups of lysine and amino endgroups of the peptide chains (*Andersen* unpublished) This indicates that the ε amino groups of lysine are free and not incorporated in any sort of linkage

Diaminopimelic acid was not detected in paper chromatography of resilin hydrolyzates Besides two bluish fluorescent spots the chromatograms only showed spots expected from the amino acid analysis by *Bailey* and *Weis Fogh* (1961) The compounds present in the fluorescent spots have now been identified (*Andersen* 1964), and all available evidence indicates that they function as cross links in resilin (*Andersen* 1963) The details are presented and discussed in the following chapters

3 Isolation and purification of the fluorescent compounds from resilin

The most suitable method for isolating the fluorescent compounds from resilin depends upon the amount of material at disposal. Paper chromatography or thin layer chromatography is to be preferred if less than 1 mg resilin is available. With larger samples column chromatography becomes advantageous. Paper chromatography has been used much during the first part of this study, since the ligaments from a single locust contain sufficient material to give well-defined fluorescent spots. It has been possible to show the presence of these compounds in dragonfly and crayfish resilin by this method. Several solvent combinations were found to give good separation, but to avoid quenching of the fluorescence only solvents without aromatic components should be used.

PAPER CHROMATOGRAPHY

The following procedure has been found to separate the two compounds from each other and from the other amino acids present in resilin (*Andersen* 1963). It involves one-dimensional descending chromatography with subsequent use of two solvents in the same direction. The first solvent is isopropanol-concentrated ammonia-water (8 : 1 : 1 v/v/v), in which both fluorescent compounds remain at the origin, while most of the other amino acids migrate. A small beaker with 3 per cent ammonia should be placed at the bottom of the chromatography jar 30–60 min before starting the chromatogram, otherwise the fluorescent compounds may migrate to some extent. When the solvent has reached the lower edge, the paper is removed, dried, and rechromatographed in the same direction in *n*-butanol-acetic acid-water (4 : 1 : 1 v/v/v) for 16–20 h. In this solvent the two fluorescent compounds have R_f values of 0.05 and 0.18. According to their position on the chromatogram they have been called Compound I and Compound II, Compound I having the smaller R_f value. The compounds are easily located by their fluorescence in ultraviolet light; the fluorescence increases when the chromatogram is exposed to

ammonia vapour whereas vapour from hydrochloric acid quenches the fluorescence almost completely Excellent separation of the fluorescent compounds is also obtained with thin layer chromatography on silica gel using the same solvent systems

COLUMN CHROMATOGRAPHY

Cellulose phosphate and DEAE-cellulose are the best materials tried so far, and they have been used routinely whereas resins as Dowex 1 and Dowex 50 are less suited because of the strong adsorption of aromatic compounds to these material (polystyrenes)

The method of purification of Compounds I and II has been modified several times and a rather simple standard procedure is given in detail

Hydrolysis Most of the resilin-containing structures contain other substances such as chitin and in some instances (tergites clypeo-labral spring tarsi etc) a contamination with hard sclerotized cuticle is inevitable Hydrolysis of the samples with 0 1 N HCl at 100° C for 6 h solubilizes all the resilin and leaves the chitin unaffected However part of the contaminating proteins may be solubilized which means that the ligaments must be carefully separated from adhering tissue and non-elastic cuticle if a hydrolysate of pure resilin is wanted Merely to obtain samples of the fluorescent compounds the other resilin-containing structures can equally well be used and excessive cleaning in unnecessary The sclerotized cuticle then present gives rise to several aromatic compounds but they are clearly separated from the fluorescent compounds during the fractionation

The partly hydrolysed resilin is further hydrolyzed to liberate the constituent amino acids The supernatant from the initial hydrolysis is added to an equal amount of concentrated HCl in a tube which is sealed and incubated at 120° C for 20 h The hydrolysate is evaporated to dryness *in vacuo* over solid NaOH

Fractionation Cellulose phosphate (Whatman P 40) is washed repeatedly with 0 2 M acetic acid poured into columns with 10 mm internal diameter and allowed to settle by means of gravity to a height of 30–50 cm The sample dissolved in distilled water is applied to the top of the column The elution is performed either with a solution of 0 5 M NaCl in 0 2 M acetic acid or by means of a gradient established by running 500 ml 1 0 M NaCl into 300–500 ml 0 2 M acetic acid in a constant volume mixing chamber The first method results in good separation of tyrosine Compound II, and Com

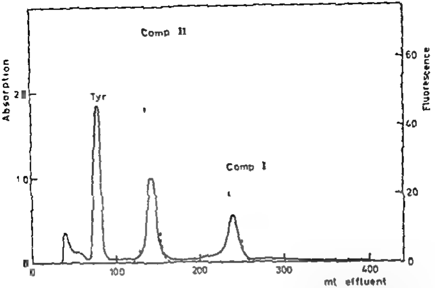


Fig 5 Fractionation of resilin hydrolysate on cellulose phosphate (1×30 cm) at room temperature Before application of the sample the column was equilibrated with 0 2 M acetic acid and elution was performed with 0 5 M NaCl in 0 2 M acetic acid Fractions of 5 ml were collected and the absorption and fluorescence were measured Unbroken line absorption at 280 mμ broken line fluorescence in arbitrary units with activation at 285 mμ

pund I eluted in this order (Fig 5) By the second method the initial elution is slower (Fig 6) which is of advantage when the sample contains hard sclerotized cuticle giving rise to several ultra violet absorbing compounds eluted between tyrosine and Compound II

The fractions containing Compounds I and II can be localized by their fluorescence or by their absorption at 280 mμ The small peak eluted after Compound I (Fig 6) represents a third fluorescent compound (Compound III) present in very small amounts Although little work has been done with respect to its structure there is no doubt that it closely resembles the two other compounds When large amounts of hydrolysed resilin were fractionated small amounts of fluorescent compounds were eluted after Compound III It cannot be decided whether they belong to the same family of compounds as the others since the amounts were too small for measurements of the absorption spectra

DEAE-cellulose can be used for fractionation of whole hydrolysates and for further purification of the fluorescent fractions obtained from the cellulose phosphate column The material (Whatman DE 50) is equilibrated with 0 02 M $NaHCO_3$ before being packed into columns of 10 mm internal diameter to a height of about 50 cm Elution is performed by means of a

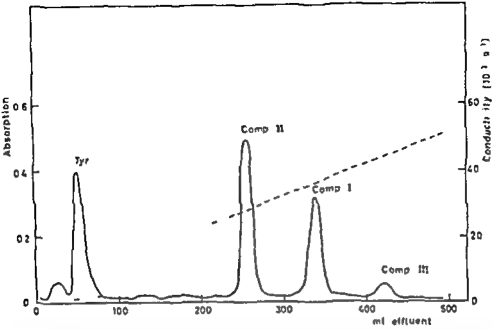


Fig 6 Fractionation of resilin hydrolysate on cellulose phosphate (1×40 cm) at room temperature Before application of the sample the column was equilibrated with 0 2 M acetic acid and elution was performed by a NaCl gradient established by running 500 ml 1 M NaCl into 300 ml 0 2 M acetic acid Fractions of 8 ml were collected and the absorption at 280 mμ and the conductivity of effluent were measured (Redrawn from *Andersen* and *Weis Fogh* 1964)

gradient established by running 500 ml 0 5 M NaCl into 500 ml 0 02 M $NaHCO_3$ Fig 7 shows the separation obtained when a hydrolysate of resilin is fractionated in this way With DEAE-cellulose the best results are obtained when the sample is salt free when applied to the column

Desalting Solutions containing the fluorescent compounds can be desalted on a column (2 × 10 cm) of cellulose phosphate in the ammonium form The sample is applied to the column in acid solution (pH below 2) and the column is then washed with several volumes of distilled water The compounds are eluted by means of 3 per cent ammonia and the effluent exhibiting the fluorescence is collected and evaporated *in vacuo* over concentrated H_2SO_4 (*Andersen* 1963)

A small column of Dowex 50 × 8 can also be used for desalting the samples The procedure is the same as for cellulose phosphate, but the Dowex resin has the advantage of a higher capacity for the fluorescent compounds On the other hand the compounds are eluted in a rather large volume due to adsorption to the resin

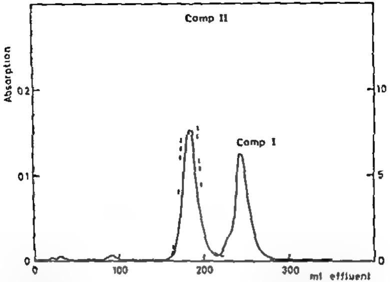


Fig 7 Fractionation of reslin hydrolysate on DEAE cellulose (1×30 cm) at room temperature Before application of the sample the column was equilibrated with 0 02 M $NaHCO_3$ and elution was performed by a NaCl gradient established by running 500 ml 0 5 M NaCl into 500 ml 0 02 M $NaHCO_3$ Fractions of 5 ml were collected and the absorption and fluorescence were measured Unbroken line absorption at 320 mμ broken line fluorescence in arbitrary units with activation at 320 mμ

Purity of the products The purification procedure thus consists of four steps (1) fractionation of hydrolysate on cellulose phosphate (2) desalting of fluorescent fractions, (3) chromatography of these fractions on columns of DEAE cellulose and (4) desalting again Usually this procedure results in pure samples of both compounds but the purity should be tested in paper chromatography with several solvents Both compounds should give a single ninhydrin positive spot the outline of which should correspond exactly to the outline of the fluorescence before spraying with the ninhydrin reagent When this criterion was not fulfilled the preparation was taken through the entire purification procedure once more The solvents used for assaying the purity were *n* butanol acetic acid water (4 1 1 v/v/v) *n* butanol formic acid (88 per cent) water (75 10 25 v/v/v) isopropanol conc ammonia water (8 1 1 v/v/v) and methylethylketone *tert* butanol water (2 1 1 v/v/v)

4 General properties of the fluorescent compounds isolated from resilin

The presence of unusual compounds in hydrolysates of resilin was first inferred from a discrepancy between the ultra violet absorption of the hydrolysate and the amount of aromatic amino acids determined by orthodox methods Inspection of paper chromatograms in ultra violet light then showed the presence of two spots with blue fluorescence, which also gave a positive reaction with ninhydrin The latter reaction was negative if the paper was pretreated with a methanolic solution of cupric nitrate (*Andersen* 1963) This indicates that the compounds are α amino acids since other amino groups do not form complexes with cupric ions stable enough to prevent the reaction with ninhydrin (*Larsen* and *Kjaer* 1960)

Compounds I and II also reacted with reagents generally used to locate phenolic compounds (*Andersen* 1963) The tests which gave positive results were Millon s reaction Folin s reaction for phenols coupling with diazotized sulphanilic acid and other diazotized amines and reaction with α nitroso-β-naphthol No colour reaction was observed after treatment with dichloroquinonechlorimide ferric chloride ammoniacal silver nitrate, and *p* dimethylaminobenzaldehyde These reactions indicate that the compounds contain a phenolic group that the *para* position to the phenolic group is not free that there cannot be more than one hydroxyl group on the same benzene ring, and that the compounds do not contain an indole nucleus Although the results of such colour tests on chromatograms must be taken with some reservation they are of value in guiding speculations with respect to possible structu[illegible]

ELECTROPHORESIS

Paper electrophoresis at different pH values (*Andersen* 1963) sho[illegible] both compounds are isoelectric between pH 4 and 5 and that the[illegible] groups ionizing near pH 2 5 (carboxylic) and near pH 9 (α amino) [illegible] ionizing near neutrality (pH 6–7) may be responsible for the [illegible] tions for phenolic groups on the chromatograms They will b[illegible]

detail later in connection with the ultra violet absorption spectra and the fluorescence

TITRATION

Solution of Compounds I and II were titrated with potassium hydroxide to determine the relative number of ionizable groups The most clearcut results were obtained for Compound II which was available in largest amounts It was found that for every one group with a pK value of 7 3 two groups are present with pK values near 2 5 and two groups with pK values near 9 9 (*Andersen* 1963) The titrations indicated the presence of groups ionizing above pH 12 but later titrations with larger amounts of material have failed to confirm this observation

The titrations are not precise at low pH values where the carboxylic groups of α amino acids ionize, making the determination of the number of these groups uncertain As Compound II has its isoelectric point near pH 5 the number of carboxylic groups must equal the number of amino groups, provided that the group ionizing at pH 7 is a phenolic group

The ionization of the carboxylic groups in amino acids can be changed to higher pH values by acylating the amino groups, making the titration of the carboxylic groups more precise Titrations of *N* methoxycarbonyl Compound II showed that the carboxylic groups have a pK value of 3 7 and that two such groups are present per group titrating near neutrality (*Andersen* 1963)

Compound I has never been obtained in sufficient quantity to permit precise titrations but the results obtained (*Andersen* 1963) indicate that, for each group with a pK of 6 2 three groups are present with pK values near 9 6 Groups are also present with pK values between 2 and 3 but their exact number cannot be determined from the titrations However the number of carboxylic groups can be assumed to equal the number of amino groups since the isoelectric point is between pH 4 and 5 Due to the scarcity of Compound I no titration of the *N* methoxycarbonyl derivative has been obtained

The titration curve for Compound I (*Andersen* 1963) indicated the presence of a group ionizing above pH 12 This has been confirmed in later titrations and the fluorescence and the absorption spectra also confirm that such a group is present

After the samples had been titrated the absorption was measured at the wavelength of maximum absorption and the molar absorption coefficient was

Table II

Molar absorption coefficients determined for fluorescent compounds from resilin at the wavelengths of maximum absorption in acid solution

Compound	Wavelength	Molar absorption coefficient
Compound I	286 mμ	8000
		9000
Compound II	283 5 mμ	5400
		5300
		5400
Methoxycarbonyl Compound II	283 5 mμ	5300

calculated (Table II) This calculation is based on the assumption that there is only one group present ionizing near neutrality It will be shown later that this assumption is correct By means of the molar absorption coefficients the concentrations of the compounds can be determined provided that no other substances are present absorbing at the wavelength of maximum absorption

The molar absorption coefficients of 8000 and 5400 for Compounds I and II have been used to determine the amounts present in resilin and thereby the degree of cross linking (*Andersen* 1963) The same values will be used in the following sections although the value for Compound I may be too low but even a value amounting to 9000 does not influence the final results much and does not affect the conclusions

QUANTITATIVE COLOUR REACTION

The titration curves indicate that Compound II contains two amino groups and two carboxylic groups for each group ionizing near neutrality and that the relative amounts of these groups are 3 3 1 in Compound I The relative number of amino groups can also be determined by measurements of the ultra violet absorption and the colour yield in the reaction with ninhydrin assuming that all α amino groups give the same colour yield with ninhydrin this is approximately true for the modification of the ninhydrin reaction used here (*Rosen* 1957) Isoleucine was used as standard and the results were calculated by means of the molar absorption coefficients given above According to this Compound II contains 1 9 amino groups and Compound I contains 2 6 amino groups (*Andersen* and *Weis Fogh* 1964)

ABSOLUTE NUMBER OF AMINO GROUPS

The absolute number of amino groups was determined by the method of *Silaev Katrukha* and *Stepanov* (1961) (*Andersen 1963*) The compound was treated with fluorodinitrobenzene in such a way that the reaction would be incomplete Assuming that all the amino groups react with fluorodinitro benzene with almost equal velocity the compound should be present in all degrees of substitution The reaction products were then separated by paper electrophoresis in an acid buffer system where unsubstituted amino groups were positively charged and carboxylic group and substituted amino groups were uncharged The unsubstituted molecules should therefore migrate fastest towards the cathode the fully substituted slowest or not at all and the partly substituted molecules at intermediate rates depending on the degree of sub stitution the number of yellow bands thus corresponding to the number of amino groups By this method Compounds I and II were shown to contain three and two amino groups respectively (Fig 8)

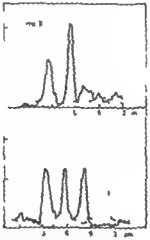


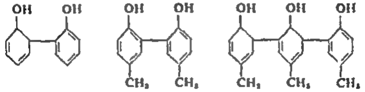
Fig 8 The reaction mixtures from partial dinitrophenylation of Compound II and Compound I separated by paper electrophoresis for about 6 h at 220 V Buffer system 85 per cent formic acid glacial acetic acid water (28 20 52 v/v/v) Fully drawn lines show the absorption at 420 mμ and broken lines the fluorescence with activation at 285 mμ All measurements are in arbitrary units (from *Andersen* 1963)

From this part of the work we can then conclude that Compound I and II are amino acids and that Compound II contains two aliphatic chains each carrying a terminal carboxylic and an α amino group whereas Compound I contains three such chains Nothing can be concluded with respect to the length of the chains or how they are connected

5 The aromatic structure of the fluorescent compounds

In this chapter the aromatic properties (ultra violet absorption, fluorescence etc) of Compounds I and II and of some model compounds will be discussed

The compounds used for comparison are 2 2 dihydroxybiphenyl (biphenol, G) 2 2 dihydroxy 5 5 dimethylbiphenyl (bicresol, H), 2,2 ,2 trihydroxy 5 5 ,5 trimethyl m terphenyl (tercresol J), two compounds (P I and P II) obtained by enzymatic peroxydation of L-tyrosine and a compound (P III) obtained by enzymatic peroxidation of pure P II In addition similar oxidation products of glycyl tyrosine were used



G H J

Biphenol was a commercial product from British Drug Houses Ltd Bicresol and tercresol were prepared by oxidation of *p*-cresol with sodium persulphate and silver nitrate according to *Bacon Grime* and *Munro* (1954) and purified by fractional precipitation from ethanol (*Pummerer Puttfarcken* and *Schopflocher* 1925) followed by recrystallization from benzene Melting points (uncorr) bicresol 155–156° C tercresol 197–198° C

L-tyrosine was oxidized by means of hydrogen peroxide and horseradish peroxidase (*Gross* and *Sizer* 1959) The reaction mixture was fractionated on cellulose phosphate (Fig 9) in the same way as hydrolysates of resilin (p 23) When Fig 9 is compared with Fig 5 it is seen that the composition of the peroxidation mixture is similar to the resilin hydrolysate with respect to fluorescent compounds The compounds from the peroxidation eluted in positions similar to Compounds II and I from resilin are called P II and P-I respectively During the fractionation P I is not completely separated from a

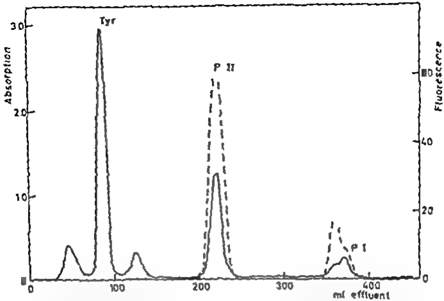


Fig 9 Fractionation of a sample of the reaction mixture after treating 0 1 g tyrosine with 5 ml 0 1 per cent hydrogen peroxide and 4 mg horseradish peroxidase (Fluka) in a total volume of 100 ml at pH 8 for 16 h at 37 C The sample was acidified to pH 2 and applied to a column of cellulose phosphate (1×50 cm) equilibrated with 0 2 M acetic acid Elution was performed as described in Fig 5 Full line absorption at 280 mμ broken line fluorescence in arbitrary units with activation at 285 mμ

third fluorescent compound which apparently has no counterpart in hydrolysates of resilin and which can be separated from P I on DEAE cellulose P I and P II can be desalted and purified by using the methods described for the compounds from resilin

Pure P II has been subjected to further oxidation by incubation with hydrogen peroxide and peroxidase This results in the formation of a new compound P III which is eluted from cellulose phosphate columns in the same position as Compound III from resilin

The N methoxycarbonyl derivatives of P I and P II as well as the corresponding derivatives from Compounds I and II were prepared according to the method described by *Bailey* (1957)

P II was esterified by refluxing a few milligrams for four hours in 15 ml absolute ethanol containing 0 5 ml conc HCl By this method a mixture of mono- and diethylester of P II was obtained They were separated on a column of cellulose phosphate equilibrated with 0 02 M sodium acetate buffer pH 5 and the elution was performed with a solution of 0 5 M NaCl in 0 02 M sodium acetate buffer pH 5 A small amount of non esterified P II emerged first indicating that this compound is uncharged at pH 5

Later the monoethylester was eluted, and the diethylester came last consistent with the assumption that this compound carries most positive charges at pH 5

The dipeptide glycyl tyrosine was also peroxidized enzymatically by the method of *Gross* and *Sizer* (1959) The reaction mixture was fractionated on cellulose phosphate as described in Fig 5, and the fluorescent fractions obtained corresponded closely to those in Fig 9 Thus the first fluorescent peak showed the same ultra violet spectrum as P II, and paper chromatography showed that glycine and P II are the only products obtained by hydrolysis of material from this peak It is therefore assumed to be glycyl P II A fluorescent compound tentatively identified as glycyl P I was also obtained, the identification being based upon the ultra violet spectra and the products obtained after hydrolysis

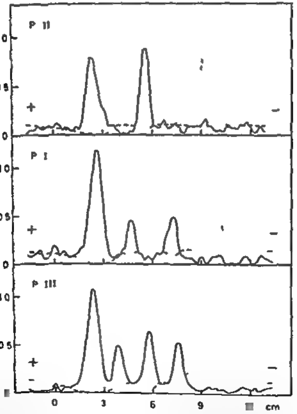


Fig 10 The reaction mixtures from partial dinitrophenylation of P I P II and P III separated as described in Fig [illegible] Full lines show the absorption at 420 mμ and broken lines show the fluorescence with activation at 285 mμ All measurements are in arbitrary units

COMPARISON BETWEEN THE RESILIN COMPOUNDS AND P COMPOUNDS

The different model compounds have been compared with the compounds obtained from resilin with respect to ultra violet absorption spectra, fluorescence activation and emission spectra the pH dependence of absorption and fluorescence, as well as their ability to form complexes with boric acid The measurements were performed as described by *Andersen* (1963)

The close similarity between the compounds from resilin and those from tyrosine peroxidation is illustrated in Fig 10, which shows the result obtained by partial dinitrophenylation of P I, P II, and P III According to these results P II must contain two amino groups P I three and P III four amino groups

Attempts were made to separate mixtures of Compound II and P II and of Compound I and P I in their components by paper chromatography in the different solvent systems (see p 26) and by column chromatography on cellulose phosphate and DEAE-cellulose It turned out to be impossible by any of these means to separate the related compounds, strongly indicating that Compound II is identical with P II and Compound I with P I

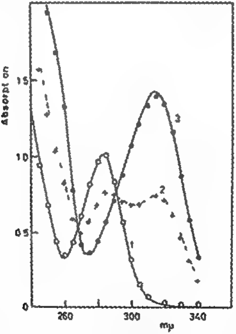


Fig 11 Absorption spectra of Compound II and P II at three pH values Full line (1) Compound II at pH 3 2 broken line (2) Compound II at pH 7 2 full line (3) Compound II at pH 11 1 Single points (O) P II at pH 3 5 (+) P II at pH 7 1 (●) P II at pH 12 0 To compare the two sets of measurements the absorption at 285 mμ in acid solution has been given the value 1 0 for both compounds

ABSORPTION SPECTRA

Compound II and P II have identical absorption spectra both at acid and alkaline reaction (Fig 11) The absorption has maximum at 283 5 mμ and 316 mμ in acid and alkaline solution respectively A minimum is present at 260 mμ in acid solution and at 274 mμ in alkaline solution Isosbestic points are present at 268 mμ and 292 mμ The corresponding spectra for biphenol and bicresol are shown in Fig 12 they are very similar to those in Fig 11, although the maxima and minima occur at slightly different wave lengths For all these compounds the absorption maximum shifts by approximately 30 mμ towards longer wavelength in going from acid to alkaline reaction With tyrosine the corresponding shift is 18 mμ in accordance with findings for monophenols

The ultra violet spectra of Compound II and P II are changed in the pH region 8 to 10 if boric acid is present in the solutions and new absorption maxima appear at 252 mμ and at 292 mμ At more acid and more alkaline

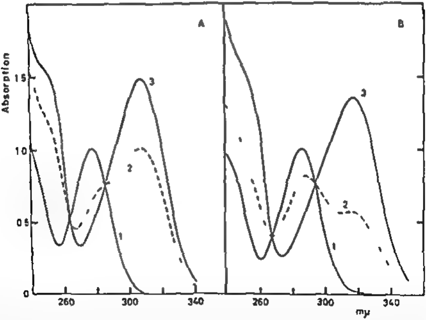


Fig 12 (A) Absorption spectra of biphenol at three pH values (1) at pH 3 1 (2) at pH 7 7 and (3) at pH 11 0 (B) Absorption spectra of bicresol at three pH values (1) at pH 3 1 (2) at pH 7 6 and (3) at pH 11 1 To compare the two sets of measurements the absorption in acid solution has been given the value 1 0 at 280 mμ (biphenol) and at 285 mμ (bicresol)

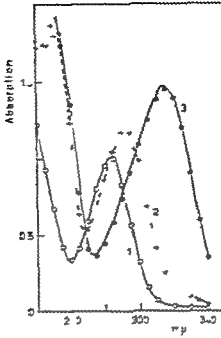


Fig. 13. Absorption spectra of Compound II and P II in 0.2 M boric acid at three pH values. Full line (1): Compound II at pH 3.7; broken line (2): Compound II at pH 7.0; full line (3): Compound II at pH 12.1. Single points: (○) P II at pH 4.0; (+) P II at pH 7.1; (●) P II at pH 12.3. To compare the two sets of measurements the absorption at 255 mμ in acid solution has been given the value 1.0 for both compounds.

reactions the spectra are unaffected (Fig. 13). Also the spectra of biphenol and bicresol are influenced by boric acid (Fig. 14). The changes indicate that some sort of complex is formed. Simple monophenols such as tyrosine do not form similar complexes whereas catechols are able to do so.

The effect of pH is shown in Fig. 15 in which the absorption of Compound II and P II is plotted as a function of pH with and without boric acid being present. The corresponding curves for biphenol and bicresol are also included. Without boric acid the ultra violet absorption changes between pH 7 and 9 for all four compounds and this is the only change in the range from pH 1 to 13. For biphenol and bicresol the change must be due to the ionization of a phenolic group since there are no other groups present which are able to ionize. Most phenolic groups ionize about pH 10 but *o,o'*-biphenols are an exception (*Musso* and *Matthies* 1951). The close similarities between these two compounds and Compound II and P II indicate that the two latter compounds also contain the *o,o'*-biphenolic structure, a notion which is supported by the ability of all these compounds to form complexes with boric acid.

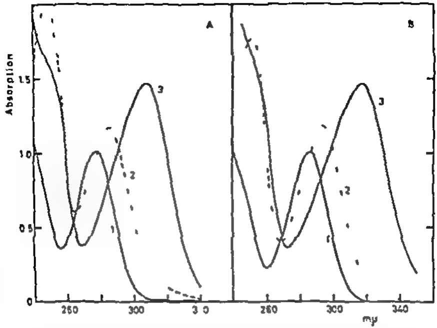


Fig 14 (A) Absorption spectra of biphenol in 0 2 M boric acid at three pH values (1) at pH 3 3 (2) at pH 7.3 and (3) at pH 12 3 (B) Absorption spectra of bicresol in 0 2 M boric acid at three pH values (1) at pH 3 0 (2) at pH 7 7 and (3) at pH 12 3 To compare the two sets of measurements the absorption in acid solution has been given the value 1 0 at 280 mμ (biphenol) and at 285 mμ (bicresol)

Compound I and P I *also have identical absorption spectra* (*Fig* 16 A) which are similar to those obtained for tercresol at corresponding pH values (Fig 16 B) In this case the absorption changes for all three compounds at two different pH values, namely between pH 6 and 8 where the absorption maximum for Compound I shifts from 286 mμ to 322 mμ, and about pH 12 where it shifts to 315 mμ (Fig 17) The shift in the alkaline region has an *isosbestic point* at 325 mμ and since the change at 325 mμ was the one originally recorded it was not observed previously (*Andersen* 1963) Both changes in absorption can be assumed to be due to phenolic groups

Compound I P I, and tercresol also form complexes with boric acid with a common absorption maximum at 292–293 mμ (Fig 18)

Pronounced similarities are therefore present between the spectra of the biphenolic group (Compound II P II biphenol and bicresol) and the terphenolic group (Compound I P I and tercresol) but significant differences are also apparent They resemble each other in having an acid phenolic group for the biphenols it ionizes between pH 7 and 8 and for the

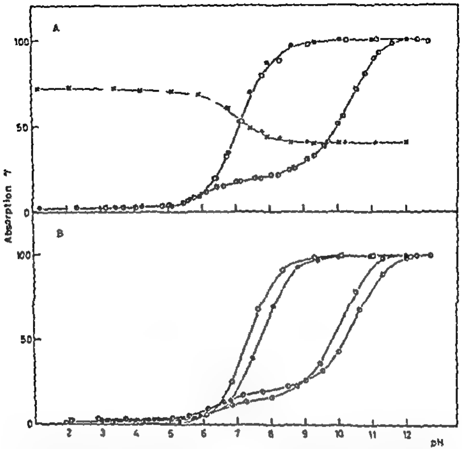


Fig 15 (A) The pH dependence of the ultra violet absorption of Compound II and P II (+) Compound II at 285 mμ (×) P II at 285 mμ (○) Compound II at 315 mμ (●) P II at 315 mμ (◐) Compound II in 0 2 M boric acid at 315 mμ (◐) P II in 0 2 M boric acid at 315 mμ

(B) The pH dependence of the ultra violet absorption of biphenol and bicresol (○) biphenol at 310 mμ (●) bicresol at 315 mμ (◐) biphenol in 0 2 M boric acid at 310 mμ (◐) bicresol in 0 2 M boric acid at 315 mμ

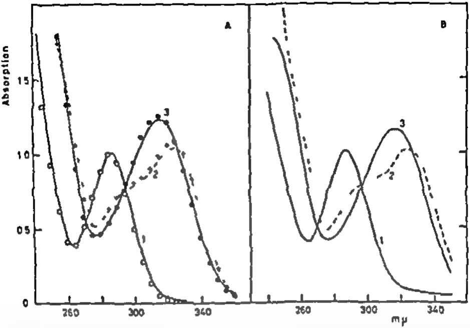


Fig 16 (A) Absorption spectra of Compound I and P I at three pH values Full line (1) Compound I at pH 2 5 broken line (2) Compound I at pH 9 7 full line (3) Compound I at pH 13 0 Single points (O) P I at pH 2 8 (+) P I at pH 9 8 (●) P I at pH 13 0 (B) Absorption spectra of tercresol at three pH values (1) at pH 2 6 (2) at pH 9 7 and (3) at pH 12 8 To compare the three sets of measurements the absorption at 285 mμ in acid solution has been given the value 1 0 for all three compounds

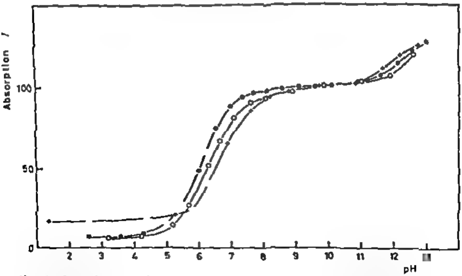


Fig 17 pH-dependence of the ultra violet absorption at 315 mμ of Compound I P I and tercresol (O) Compound I (●) P I (+) tercresol The absorption values are expressed relatively to the absorption measured near pH 10

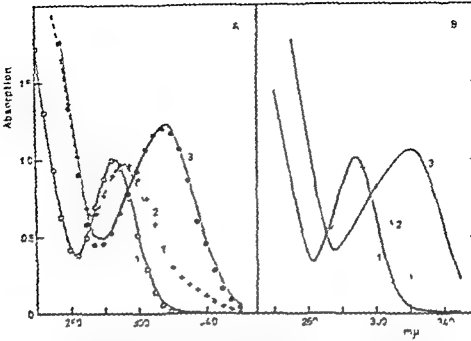


Fig 18 (A) Absorption spectra of Compound I and P I in 0 2 M boric acid at three pH-values Full line (1 Compound I at pH 2.2 broken line (2) Compound I at pH 6.2 full line (3) Compound I at pH 12.8 Single points (O) P I at pH 2 1 (+) P I at pH 6.3 (●) P I at pH 12.8 (B) Absorption spectra of tercresol in 0 2 M boric acid at three pH-values (1) at pH 2.4 (2) at pH 6 4 and (3) at pH 12 4 To compare the three sets of measurements the absorption at 285 mμ in acid solution has been given the value 1 0 for all three compounds

terphenols between pH 6 and 7 For both groups of compounds the wave length of maximum absorption is shifted about 30 mμ towards the red when the phenolic group ionizes and both groups form similar complexes with boric acid The differences in spectral behaviour between the two types can be explained by the ionization of a second phenolic group in the terphenols having a pK value of about 12

Compound III from resin the compound eluted after Compound I from cellulose phosphate shows ultra violet absorption (Fig 19) resembling that of the other compounds and identical with the spectrum of P III, the compound formed when P II is enzymatically peroxidized. The pH-dependence of the absorption of these two are also similar and Fig 20 shows that the absorption changes in two steps at pH 3 5 and at pH 8 5

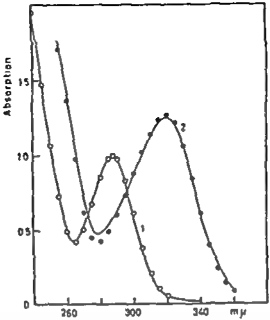


Fig 19 Absorption spectra of Compound III and P III at two pH values Full line (1) Compound III at pH 2 full line (2) Compound III at pH 13 Single points (O) P III at pH 2 (●) P III at pH 13 To compare the two sets of measurements the absorption at 285 mμ in acid solution has been given the value 1 0 for both compounds

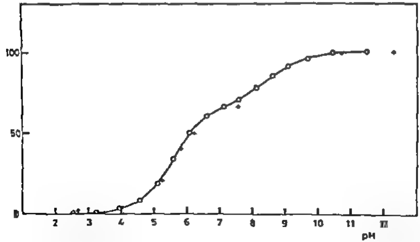


Fig 20 pH-dependence of the ultra violet absorption at 325 mμ of Compound III and P III (+) Compound III (O) P III The values are expressed relatively to the highest absorption value measured

FLUORESCENCE EMISSION

The biphenolic and the terphenolic' compounds have almost identical fluorescence emission spectra (Fig 21) Maximum emission occurs between 410 and 415 mμ, with biphenol as the only exception (400 mμ) Pieces of native resilin show a fluorescence emission resembling those in Fig 21 but with maximum at 420 mμ (*Andersen* 1963) For all the compounds the fluorescence is stronger in alkaline than in acid solution but the emission maximum does not change

P II has a higher fluorescent yield than P I (Fig 22) also when calculated on a molar basis The figure also shows that there is a considerable self quenching at higher concentrations

The compounds are unstable when exposed to strong ultra violet light especially in alkaline solution This decomposition results in a decrease in fluorescence which eventually disappears completely Due to the decomposition it was necessary to perform the measurements of the emission as fast as possible since even a few minutes exposure to ultra violet light may result in a decrease in fluorescence During measurements of the fluorescence activa

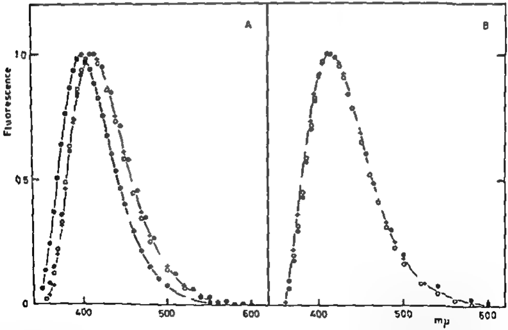


Fig 21 (A) Spectra of fluorescent light emitted from Compound II (+) P II (O) biphenol (◑) and bicresol (●) all at alkaline reaction Activation at 253 7 mμ (B) Spectra of fluorescent light emitted from Compound I (+) P I (O) and tercresol (●) all at alkaline reaction Activation at 253 7 mμ

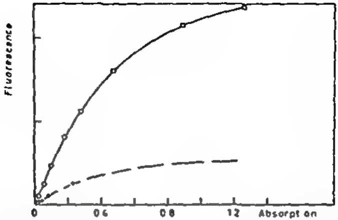


Fig 22 Fluorescent yield of P I (+) and P II (O) at pH 4 Absorption measurements and fluorescence activation were performed at the wavelength of maximum absorption 283 5 mμ in the case of P II and 286 mμ in the case of P I

tion the samples were exposed to light intensities so weak that virtually no decomposition could be observed

FLUORESCENCE ACTIVATION

Fig 23 shows the activation spectra of Compounds I and II in acid and alkaline solutions The spectra resemble the absorption spectra with respect to maxima and minima However the fluorescence appears relatively weak at the shorter wavelengths because the hydrogen lamp used for the activation has a higher light intensity at longer than at shorter wavelengths

Fig 24 shows the pH dependence of the fluorescence activation of the biphenolic compounds with and without boric acid By comparing Figs 23 B and 24 it is seen that the fluorescence of the biphenols changes between pH 7 and 8 and that the change is connected with an alteration of the activation spectrum The changes occur at the same pH as the shift in the absorption spectra from the acid to the alkaline form (Fig 15) so that the alteration in activation and absorption must be due to the ionization of the same group

The intensity of the fluorescence of Compound II and P II changes between pH 1 and 3 when activated at 285 mμ corresponding to the change in the fluorescence of tyrosine (*White* 1959) For tyrosine the decrease in fluorescence occurs at pH 2 5 and is believed to be connected with the neutralization of the carboxylic group A similar mechanism can be assumed for Compound II and P II since the change in fluorescent yield occurs between pH

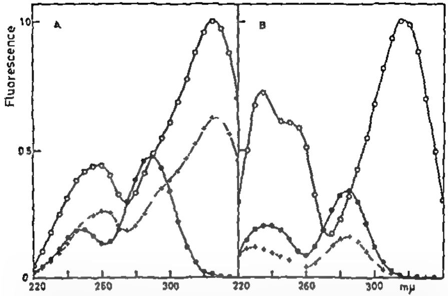


Fig 23 (A) Activation spectra for Compound I at three pH values (●) at pH 3 4 (O) at pH 7 7 and (+) at pH 11 4 (B) Activation spectra for Compound II at three pH values (+) at pH 1 2 (●) at pH 4 1 and (O) at pH 9 4
No corrections were attempted although the intensity of the activating light was higher at the longer wavelengths than at the shorter

3 and 4 after acetylation of the α amino groups, i e at an acidity corresponding to that of the carboxylic groups in such derivatives For the mono and diethylester of P II the yield of fluorescence is constant between pH 1 and 5 indicating that two free carboyxlic groups are required to produce the decrease in fluorescence

The fluorescence of bicresol decreases below pH 2 corresponding to the decrease described for phenol at about pH 0 (*White* 1959) The decrease is believed to be due to the quenching effect of the hydrogen ions in the medium

In contrast to what is found for Compound II and P II the state of ionization of the carboxylic groups has no influence on the fluorescence of Compound I and P I (Fig 25) On the other hand, the fluorescence of these compounds is influenced by the ionization of the amino groups The decrease in fluorescence which occurs about pH 9 is not observed when the amino groups are blocked such as in methoxycarbonyl Compound I and in glycyl P I, and neither is it present in tercresol It is not known whether all three amino groups or only one or two have to be free to give this change in fluorescence

The fluorescence of the terphenolic compounds also differs from that of the biphenolic compounds in being completely abolished above pH 13

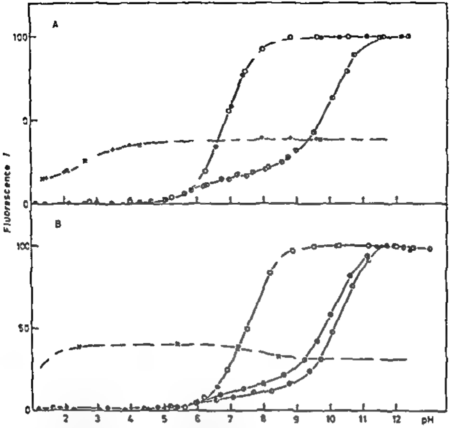


Fig 24 (A) pH-dependence of the fluorescence of Compound II and P II (+) *Compound II activated at 285 mμ* (×) *P II activated at 285 mμ* (O) Compound II activated at 315 mμ (●) P II activated at 315 mμ (◑) Compound II activated at 315 mμ in 0 2 M boric acid (◐) P II activated at 315 mμ in 0 2 M boric acid (B) pH-dependence of the fluorescence of biphenol and bicresol (O) biphenol activated at 310 mμ (●) bicresol activated at 315 mμ (◐) biphenol activated at 310 mμ in 0 2 M boric acid (◑) bicresol activated at 315 mμ in 0 2 M boric acid (×) bicresol activated at 285 mμ.

This quenching seems to be connected with the dissociation of a second phenolic group the presence of such a group was also indicated by the change in ultra violet absorption between pH 12 and 13 (Figs 16 and 17)

The fluorescence of these compounds is influenced by the presence of boric acid in the medium as illustrated for the biphenolic compounds in Fig 24

DISCUSSION

Mild oxidation of *para* substituted monophenols may result in the formation of bi and terphenols as first demonstrated by *Pummerer Melamed* and

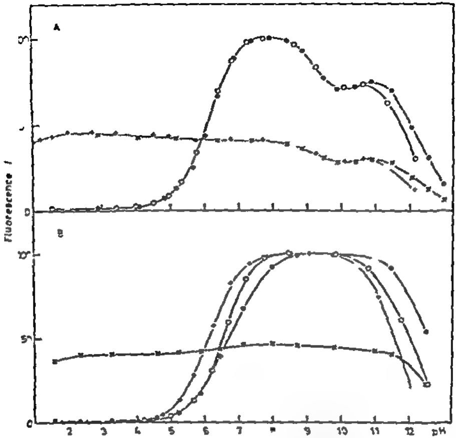
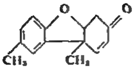
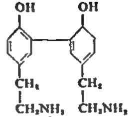


Fig 25 (A) pH-dependence of the fluorescence of Compound I and P I (+) Compound I activated at 285 mμ (×) P I activated at 285 mμ (O) Compound I activated at 325 mμ (●) P I activated at 325 mμ. (B) pH-dependence of the fluorescence of tercresol N-methoxycarbonyl Compound I and glycyl P I (×) tercresol activated at 285 mμ (●) tercresol activated at 325 mμ (O) methoxycarbonyl Compound I activated at 325 mμ (+) glycyl-P I activated at 325 mμ.

Puttfarcken (1922) who obtained bicresol (H) by oxidation of *p*-cresol with alkaline potassium ferricyanide. Other products were also formed during this reaction, such as a ketone having the structure (K) established by *Barton et al* (1955) and a phenolic compound which *Westerfeld* and *Lowe* (1942) suggested to be tercresol (J). These products can be obtained from *p*-cresol by means of a wide range of oxidants, hydrogen peroxide together with the enzyme peroxidase being used by *Westerfeld* and *Lowe* (1942). The same system was used by *Gross* and *Sizer* (1955, 1959) for the oxidation of tyrosine and tyramine. From the reaction mixtures dityrosine (E) and dityramine (L) could be isolated, and it was suggested that trimers and higher polymers were formed as well.

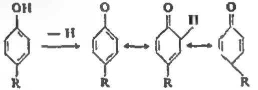


K

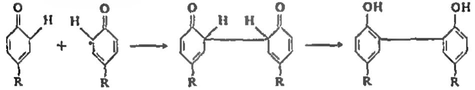


L

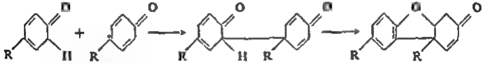
Free radicals are thought to be intermediates in the oxidative formation of biphenols from monophenols (*Barton* and *Cohen* 1957 *Erdtman* and *Wachtmeister* 1957 *Musso* 1963)



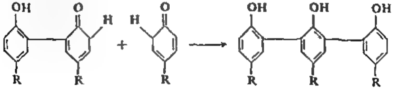
Different dimers can be formed by pairing of these radicals but the formation of C–C bonds is generally the dominant reaction (*Barton* and *Cohen* 1957) Further rearrangements can then lead to biphenol



or to Pummerer s ketone

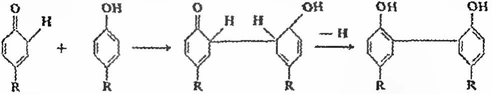


These dimers can also be oxidized to free radicals and undergo further reactions explaining the formation of trimers and higher polymers



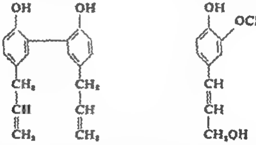
The endproduct could also be formed by another route Instead of two radicals reacting with each other a free radical could react with an unoxidized

molecule, and the product of this reaction could then be oxidized to the final product (*Barton* and *Cohen* 1957)



The radical pairing hypothesis is generally favoured since stable radicals can be obtained by the oxidation of some phenols In solution these radicals exist in equilibrium with the corresponding dimers (*Goldschmidt* and *Schmidt* 1922)

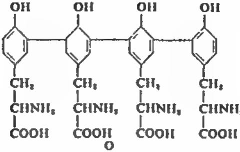
A wide range of different compounds containing the biphenolic structure has been isolated, mainly from plants One of the simplest is magnolol (M) occuring in the bark of certain *Magnolia* species (*Sugu* 1930) but also such a complex material as lignin contains biphenolic groupings (*Aulin Erdtman* 1958, *Pew* 1963) Many problems regarding the structure and biosynthesis of lignin are still unsolved, but the process is believed to involve the condensation of free radicals derived from a phenylpropane unit such as for instance coniferylalcohol (N) (*Brown* 1964) Due to the double bond in the side chain the free radicals can react in several ways other than those possible for the radicals derived from simple phenols This makes the final products very complicated

M  N

P II is so similar to biphenol and bicresol with respect to fluorescence and ultra violet absorption that there can be no doubt that P II contains the biphenolic structure Furthermore Compound II from resilin appears identical with P II in all respects so these two compounds must be one and the same, dityrosine (E) The result confirms the observation of *Gross* and *Sizer* (1955 1959) that the main product from the enzymatic peroxidation of tyrosine is dityrosine

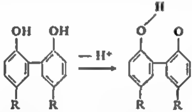
P I and Compound I also appear to be identical and since both contain three amino groups and are very similar to tercresol with respect to fluorescence and absorption they can be assumed to be trityrosine (F)

Compound III from resilin may have the quarter phenolic structure te tratyrosine (O) This is supported by the observation that compound P III formed during enzymatic peroxidation of dityrosine, has the same absorption spectrum and pH dependence of absorption as the resilin compound The structure is also in accordance with the finding that P III contains four amino groups



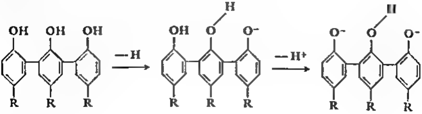
O

The structures proposed for Compounds I and II (tri and dityrosine) are consistent with the properties of these compounds and they explain the un usual acidity of the phenolic groups which for a long time was a very puzzling observation As already mentioned this acidity is a feature of *o o* biphe nols and is due to the formation of a hydrogen bond between the unionized and the ionized phenolic group (*Musso* and *Matthies* 1961)



This causes the first phenolic group to ionize easily whereas the second group becomes a weaker acid According to *Musso* and *Matthies* (1961) bi phenol has a pK_1 of 7 5 and a pK_2 above 13 This corresponds to the results for Compound II where a phenolic ionization is observed at pH 7 2 and where no further changes in ultra violet absorption can be observed below pH 13

Compound I shows phenolic ionizations both at pH [illegible] and pH 12 and these ionizations may be represented thus



Of these structures, the second should fluoresce strongest, and the third should be nonfluorescent, according to Fig 25 A

The final proof for the structures proposed here would be the synthesis of the compounds by means of well established chemical methods To some extent this has been established in the case of P II (*Gross* and *Sizer* 1955) They compared the chemically synthesized dityrosine with the main compound from the enzymatic peroxidation of tyrosine and it was found that the two compounds had identical R_f values when chromatographed on paper

6 Evidence for Compounds I and II as cross-links in resilin

The structures proposed for the two fluorescent compounds (di- and trityrosine) are in accordance with their function as cross-links in the protein. The amino groups as well as the carboxylic groups should therefore be incorporated in some sort of linkage in the protein chains, the bonds presumably being peptide linkages.

In order to demonstrate beyond doubt that di- and trityrosine function as cross-links it is necessary to show (1) that their amino groups are incorporated in some sort of linkage in the native protein and (2) that this also applies to the carboxylic groups. (3) From partial hydrolysates of resilin it should be possible to isolate peptides containing fluorescent amino acids and having two (three) amino end groups and two (three) carboxylic groups. (4) Finally one must demonstrate that the different peptide chains in such a cross-linked peptide originate from different protein chains in the native material.

It would be a formidable task to demonstrate all four points, especially the last one, since it demands a complete amino acid sequence analysis. But if the first three points could be established there could hardly be room for any doubt, di- and trityrosine could then be considered to be the cross-links in resilin.

AMINO GROUPS

If some of the amino groups of di- and trityrosine were free in native resilin they would be expected to react with dinitrofluorobenzene at slightly alkaline reaction. When resilin was treated this way (*Andersen* 1963) the ε-amino group of lysine residues and the phenolic group of tyrosine residues reacted freely, indicating that both are readily accessible for the reagent. Small amounts of DNP-serine and DNP-glutamic acid (or DNP-aspartic acid) were also formed, presumably they originate from the amino end groups of the peptide chains. The amount of these end groups indicate an average molecular weight of the individual peptide chains of about 100 000, but this

value can only be approximate due to the considerable corrections for the losses occurring during hydrolysis and chromatography

There was no indication of the formation of *N* substituted di and trityrosine during the dinitrophenylation Two derivatives were formed which were assumed to be *O* DNP di and trityrosine, since they resembled *O* DNP tyrosine in ultra violet absorption and were ninhydrin positive

The reaction with ninhydrin has been used to determine the state of the amino groups of di and trityrosine in native resilin Both fluorescent amino acids could be isolated unchanged after hydrolysis of the ninhydrin treated ligaments indicating that their amino groups are not free in the untreated protein (*Andersen* and *Weis Fogh* 1964)

Fig 25 shows that the fluorescence of Compound I decreases at pH 9 when the amino groups of this compound are free Samples of resilin have been digested with proteolytic enzymes, whereby a mixture of fluorescent and non fluorescent peptides is obtained When the pH dependence of the fluorescence of such a digest was determined (Fig 26) no decrease was found in the fluorescence at pH 9, whereas the decrease due to the second phenolic ionization in trityrosine is present at pH 12 (*Andersen* and *Weis Fogh* 1964) This indicates that at least some of the amino groups in trityrosine are involved in some sort of linkage in the digestion mixture This was presumably also the

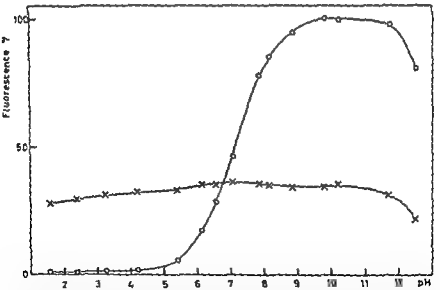


Fig 26 pH-dependence of the fluorescence of resilin digested with the protease subtilisin for 25 h at 30 C and pH 6 0 This treatment resulted in complete solubilization of the resilin (×) activated at 285 mμ (O) activated at 320 mμ (Redrawn from *Andersen* and *Weis Fogh* 1964)

case in the undigested protein although transpeptidation may have occurred during the proteolysis

The combined evidence from these different ways of investigating the state of the amino groups of di and trityrosine demonstrates clearly that these groups are involved in some sort of linkage in resilin The experiments do not show whether they are peptide linked or whether other bonds are involved However the lability of the linkages towards acid and alkali indicates that peptide linkages are involved (*Andersen* 1963)

CARBOXYLIC GROUPS

Fig 24 shows that the fluorescence of dityrosine changes between pH 2 and 3 which must be due to the ionization of the carboxylic groups This change in fluorescence is not observed in the peptide mixture from the enzymatic digestion of resilin (Fig 26) indicating that at least one of the carboxylic groups of dityrosine is combined in these peptides and presumably also in undigested resilin

Hydrazinolysis of resilin was performed according to *Akabori et al* (1953) by heating a sample of resilin with anhydrous hydrazine to 100°C for 6 hours This treatment should split the peptide bonds with the formation of amino acid hydrazides from all amino acid residues except those in the C-terminal position being liberated as free amino acids After removal of excess of hydrazine by evaporation *in vacuo* over concentrated H_2SO_4 the reaction product was subjected to paper chromatography in butanol acetic acid water (4 1 1 v/v/v) and in isopropanol conc ammonia water (8 1 1 v/v/v) None of these chromatograms showed di and trityrosine In paper electrophoresis at pH 5 of the reaction mixture the fluorescence migrated towards the cathode whereas control samples of free di and trityrosine hardly moved This indicates that at least some of the carboxylic groups in the fluorescent compounds are present in the form of hydrazides which leads to the conclusion that they were peptide bound in resilin The result does not show that *all* carboxylic groups were peptide bound since electrophoresis and chromatography only can be used to differentiate between the possible mono-, di and trihydrazides of the two compounds when these derivatives have been prepared synthetically for comparison

This means that the state of the carboxylic groups of di- and trityrosine in resilin has not been established with the same degree of certainty as the state of the amino groups The evidence available indicates, however that most of these groups are not free in the protein but are involved in peptide linkage

DISCUSSION

Strictly speaking the evidence so far obtained is insufficient to prove that the fluorescent amino acids function as cross links in resilin although it strongly indicates that this is the case The isolation in the pure state of a cross linked peptide has not been performed as yet although there is no doubt that such peptides are liberated during enzymatic hydrolysis of resilin

However the idea that the fluorescent amino acids are in fact acting as cross links has been confirmed by measurements of the amounts of the two compounds in resilin (*Andersen* 1963 *Andersen* unpublished) The analyses were performed by column fractionation of hydrolysates of carefully purified resilin samples of known weight whereafter the absorption of the different fractions was measured at the wavelength of maximum absorption The amounts of tyrosine di and trityrosine were calculated by means of the molar

Table III

Number of residues of tyrosine dityrosine and trityrosine in 10^5 g locust resilin and the average molecular weight of peptide chains between two neighbouring cross links

Conditions and material	Amount of resilin mg	Tyrosine	Dityrosine	Trityrosine	Sum of tyrosine residues	Chain weight
Standard material						
normal conditions wing hinges	7 38	26 1	8 8	3 8	55 1	3450
normal conditions prealar arms	7 11	24 5	7 9	3 9	51 8	3640
normal conditions both ligaments	12 88	24 5	9.5	3 7	54 4	3320
normal conditions[1]) prealar arms	[illegible] 98	–	9 2	4 2	–	3220
normal conditions[1]) wing hinges	2 78	–	8 8	6 1	–	2790
normal conditions[1]) wing hinges	7 71	–	9 3	5 1	–	2950
Special material						
constant light 38 C both ligaments	1 80	25 8	9 3	2 8	52 6	3700
constant darkness 38 C both ligaments	1 74	25 8	9 0	2 6	51 7	3880
constant darkness 28 C both ligaments	8 39	30 2	9 0	4 3	61 1	3240
normal conditions pre imaginal prealar arms	3 40	33 7	7 1	3 9	59 6	3860

[1]) From [illegible] 1963

absorption coefficients Table III gives the results together with the average chain weight between two cross links The latter figure was calculated on the assumption that dityrosine connects two peptide chains and trityrosine three chains As mentioned earlier (p 19) the average chain weight has been estimated from the physical analysis to be about 5100 in dragonfly resilin and about 3400 in locust resilin The amounts of the two compounds in locust resilin correspond closely to the expected degree of cross linking The close agreement between the two completely independent estimates of the distance along the peptide chain between two neighbouring junction points indicates that the network must be fairly regular and that physical entanglements can play only a minor role as they would influence the physical but not the chemical determinations

The number of analyses is too small to permit reliable conclusions with respect to the variations in the amount of the different tyrosine derivatives, but certain trends can be noticed which may be significant The tyrosine content appears to be higher in resilin from animals grown at constant low temperature (28°C) or in the pre imaginal material than in the standard material (standard conditions alternating 12 h light at 35°C and 12 h darkness at 25°C) The content of dityrosine is low in one of the analyses of standard material but otherwise, it appears to be constant with the exception of the preimaginal material The resilin from animals grown at constant high temperature (38°C) has a lower content of trityrosine than the standard material but even in the latter the content of this amino acid shows appreciable variation The total content of tyrosine units present in the three amino acids is rather constant However the figures are lower for the two samples from animals grown at constant high temperature, and the two samples with the highest content are from animals grown at low temperature or from material deposited before the final moult

It has been found (*Neville* 1963 a *Kristensen* in press) that resilin deposited during day conditions (35°C and light) shows stronger fluorescence than resilin deposited during night conditions (25°C and darkness) resulting in a banded appearance of sections in the fluorescense microscope (Fig 2) The material deposited before the final moult shows weaker fluorescence than the material deposited afterwards These differences in fluorescence indicate a difference in the content of fluorescent amino acids and therefore also in the degree of cross linking Since dityrosine shows a much stronger fluorescence than trityrosine (Fig 22) the results in Table III are in accordance with the observed differences in fluorescence However it appears strange that the total content of tyrosine units is not constant since it should be a measure of the tyrosine content of the non cross linked precursor

of resilin, proresilin Possible explinations which can be proposed are (1) The observed variations are not real but are due to inaccurate determinations In this case, however the figures would be expected to vary at random (2) Tyrosine residues in proresilin can give rise not only to di and trityrosine residues but also to small amounts of compounds which have not been deter mined in the analyses (tetratyrosine) During the enzymatic peroxidation of tyrosine *in vitro* products were obtained for which no counterparts have been found in resilin so far and it is possible that they are present in small amounts (3) It is possible, although not very likely that small differences in amino acid composition may exist in proresilin produced under different external conditions

7 Biosynthesis of the cross-links in resilin

Only a few experiments have been performed with relation to the formation of cross links in resilin. Available facts will be presented and discussed in this chapter as a basis for a hypothesis.

DEPOSITION OF RESILIN

Neville (1963 a, b) investigated the time course of the deposition of resilin and chitin in the locust ligaments. With respect to resilin he found that the most active phase was during the first 3–4 days after the final moult and that the rate of deposition then decreased slowly and finally stopped 2–3 weeks after the moult. Some deposition also occurs during the last three days before the moult.

There is a direct proportionality between the amount of resilin deposited up to a given age and the amount of fluorescent material present (*Neville* 1963 b). The proportionality indicates that the density of the cross links remains constant throughout the period of deposition, but slight differences between the pre- and post-imaginal material and between day- and night-material would not have been observed in a determination of this kind.

The time lag between the secretion of a soluble hypothetical precursor of resilin, proresilin, and the cross linking must be small. Autoradiographs of sections of ligaments from animals which have received injections of tritium labelled tyrosine shows that there is only an interval of a few hours between a single injection of radioactive tyrosine and its incorporation into cross links (*Kristensen* in press).

PRECURSORS FOR THE CROSS LINKS

Locusts were injected with ^{14}C-labelled amino acids (phenylalanine, tyrosine) at different times during adult development (*Andersen* and *Kristensen* 1963 and unpublished). After the animals had matured (3 weeks old) they were killed, the ligaments dissected out, hydrolysed and the distribution of

the activity between the different amino acids in the hydrolysates was determined In animals receiving ^{14}C-labelled phenylalanine the activity was restricted to phenylalanine tyrosine and di and trityrosine After injection of ^{14}C labelled tyrosine, activity was present only in tyrosine and in di and trityrosine but not in phenylalanine or any of the other amino acids in resilin These findings indicate that di and trityrosine are synthesized from tyrosine originating either from tyrosine in the food or from hydroxylation of phenylalanine

phenylalanine → tyrosine → di and trityrosine

The experiments did not show whether protein bound tyrosine is transformed to dimers and trimers or whether these compounds are formed from free tyrosine and then incorporated into the protein The first process appears to be the simplest and it has now been possible to transform protein bound tyrosine into di and trityrosine *in vitro* by an enzymatic process (see p 59) which may occur *in vivo*

The experiments with radioactive precursors gave a peculiar result which we are not able to explain fully at the moment When the specific activities (counts per min per μmole) were calculated both for di and trityrosine and for the tyrosine isolated from resilin it was found that dityrosine had a specific activity almost three times as high as that of the isolated tyrosine and that the specific activity of trityrosine was between five and six times higher than that of tyrosine We were inclined to believe that three molecules of tyrosine were used for the synthesis of one molecule of Compound II and five or six molecules for Compound I However, an unequal distribution of tyrosine and the two compounds in the different parts of the ligaments could give rise to the observed ratios Thus if there are relatively more tyrosine residues in the preimaginal resilin than in the postimaginal then in the whole ligament the relative specific activity of tyrosine will appear to be too low, and the relative specific activity of di and trityrosine too high, because all injections of radioactive amino acids were done after the final moult restricting the incorporation to postimaginal resilin The amounts in preimaginal resilin of tyrosine di and trityrosine given in Table III indicate such an unequal distribution The variations are of the right order of magnitude to explain the discrepancies but more material is needed before such an explanation can be accepted However it seems safe to conclude that di and trityrosine are formed from two and three tyrosine residues respectively

ARTIFICIAL CROSS LINKING OF PROTEINS

When most proteins are treated with hydrogen peroxide and peroxidase some of the tyrosine residues become oxidized but apparently di and trityro

sine are not formed (*Sizer* 1953, *Gross* and *Sizer* 1959) With this treatment I have obtained formation of di- and trityrosine in significant amounts in only a single protein silk fibroin Under similar conditions bovine serum albumin and casein gave rise to only very small amounts of these products

Silk fibroin was dissolved by means of concentrated LiSCN (70 per cent) and dialyzed against several changes of distilled water to remove the thiocyanate ions At this stage fibroin remains in solution but in the course of some days a gel is formed The gel can be dissolved again in concentrated solutions of LiSCN or in cupric ethylenediamine A solution of horseradish peroxidase and hydrogen peroxide was added to the dialyzed solution of silk fibroin and the mixture incubated at 37° C for 16 h In the course of a few minutes the solution changed to a rigid gel showing blue fluorescence when exposed to ultra violet light Pieces of this gel could not be redissolved by means of solutions of LiSCN or cupric ethylenediamine although they swelled considerably in these media When the pieces were transferred back to water they soon returned to their former size and shape Pieces swollen in water were

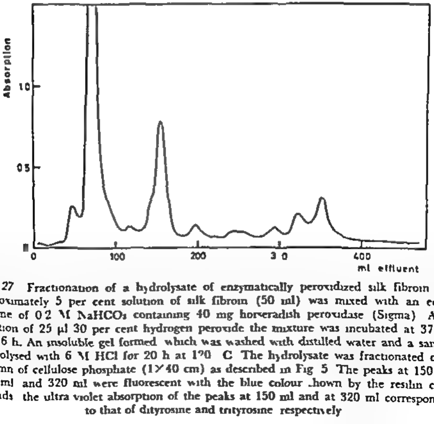


Fig 27 Fractionation of a hydrolysate of enzymatically peroxidized silk fibroin An approximately 5 per cent solution of silk fibroin (50 ml) was mixed with an equal volume of 0.2 M $NaHCO_3$ containing 40 mg horseradish peroxidase (Sigma) After addition of 25 µl 30 per cent hydrogen peroxide the mixture was incubated at 37 C for 16 h. An insoluble gel formed which was washed with distilled water and a sample hydrolysed with 6 M HCl for 20 h at 120 C The hydrolysate was fractionated on a column of cellulose phosphate (1×40 cm) as described in Fig 5 The peaks at 150 ml 290 ml and 320 ml were fluorescent with the blue colour shown by the resilin compounds the ultra violet absorption of the peaks at 150 ml and at 320 ml corresponded to that of dityrosine and trityrosine respectively

isotropic but became birefringent when compressed by means of forceps During these manipulations they easily broke but if they withstood the treatment they returned to their former shape when the force was removed

This suggests that cross links have been established between the peptide chains in the fibroin and that the protein gel formed in this way can be considered a model system for resilin This concept is confirmed by the presence of di and trityrosine in hydrolysates of the gel (Fig 27) The amino acids are not present in hydrolysates of the untreated protein

It is interesting that silk fibroin in contrast to other proteins can be cross linked by peroxidation of the tyrosine residues *Earland* and *Stell* (1957) have found that silk fibroin becomes insoluble after oxidation with such oxidants as chlorine chlorine dioxide and potassium permanganate They suggested that some sort of cross linking occurs during the process and they gave evidence that intact tyrosine residues are necessary for the reaction to occur but the nature of the postulated cross links was not elucidated It cannot be excluded that they consist of di and trityrosine

Silk fibroin is an exceptional protein in several respects It contains a very high percentage of amino acids with very short side chains such as glycine and alanine it does not contain any tryptophan or sulphur containing amino acids and the tyrosine content is relatively high All these features can be expected to make cross linking between tyrosine residues easier partly for steric reasons and partly because tyrosine is the only amino acid present susceptible to oxidation by means of peroxidase (*Sizer* 1953)

These properties which may be essential for the cross linking of silk fibroin are also assumed to be present in proresilin the hypothetical precursor of resilin This precursor corresponds to the non-cross linked peptide chains of resilin and should have the same amino acid composition as resilin except that proresilin should not contain any di and trityrosine and the tyrosine content should be correspondingly higher According to Table III about 5–6 residues per 100 should be tyrosine in proresilin A main difference between silk fibroin and proresilin should therefore be that the former only contains few amino acid residues with charged groups on the sidechains It is reasonable to assume that if a precursor of resilin exists it should also be susceptible to oxidation by means of the hydrogen peroxide peroxidase system resulting in the formation of di and trityrosine residues

DISCUSSION OF THE CROSS LINKING PROCESS IN RESILIN

A scheme for the biosynthesis of resilin has been suggested (*Andersen* and *Weis Fogh* 1964) Since then the cross links of resilin have been identified

making possible a more complete scheme without altering its essential features

The main points of the scheme are

(1) *Synthesis of proresilin*

The non cross linked peptide chains of resilin called proresilin are synthesized on the ribosomes of the cells by the normal route for biosynthesis of proteins

(2) *Storage*

Before being secreted proresilin is stored in vacuoles in the cells Vacuoles are abundant in the cells during the period of resilin deposition, and their content is non fluorescent However we have no evidence that it consists of proresilin

(3) *Secretion*

During deposition of resilin the soluble proresilin is liberated from the cells

(4) *Activation*

Some of the tyrosine residues in proresilin are oxidized to free radicals during or immediately after the secretion

(5) *Cross linking*

When two such radicals come close enough together they react with each other and form a dityrosine residue If the two tyrosine radicals are located in different peptide chains the dityrosine residue formed will function as a cross-link, and if they are located in the same peptide chain the result will be a loop on the chain

I shall restrict the discussion to the two last points as the problems raised by the other three fall outside the scope of this work

The formation of dityrosine from tyrosine is an oxidative process and it is reasonable to assume that it also occurs *in vivo* through the formation of free radicals For the moment however there is no evidence that free radicals are formed during the deposition of resilin The oxidation of tyrosine residues in proresilin is probably an enzymatic process and since peroxidase together with hydrogen peroxide is able to perform such an oxidation of protein bound tyrosine *in vitro* it is likely that it is also a peroxidase which oxidizes proresilin *in vivo* However the enzyme need not belong to this group and other substrates than hydrogen peroxide could function as the hydrogen acceptor

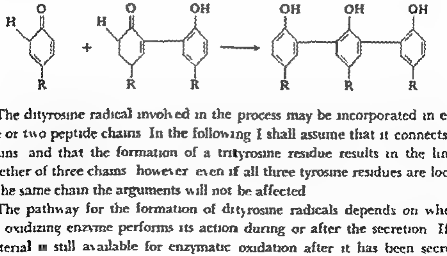
For the moment it is not possible to decide where the oxidation to free radicals occurs One possibility is that it takes place immediately *before* the

chains are liberated by the cells but this seems rather unlikely as the free radicals are very reactive and it might have undesirable side effects if they were formed inside the cells with the possibility of reacting with other cell components

Another possibility is that the enzyme which oxidizes (peroxidizes) the tyrosine residues is located near to or in the cell membrane in such a way that the chains are oxidized *while* they are being secreted and that no further oxidation can occur after they have left the cells In this case there should be a narrow zone between the cell membrane and the fully cross linked resilin This zone should contain proresilin in the oxidized but non cross linked or partly cross linked state and this proresilin should be rapidly transformed to resilin as the dimerization of the tyrosine radicals progress Free radicals will be present on the surface of the resilin which is therefore unsaturated with respect to cross links and these free radicals will react with the continuously secreted oxidized proresilin resulting in a steady growth of the ligament

A similar result may be obtained if unmodified proresilin is secreted into the growth zone and becomes oxidized *after* it has left the cells instead of being oxidized during the secretion The oxidizing enzyme would then have either to be secreted into the growth zone and perform its action there or to be localized on the outside of the cell membrane so that the secreted proresilin will be in constant contact with the enzyme If the enzyme is secreted it must be expected to be incorporated in the final resilin as it will become captured in the network formed This would of course not happen if the enzyme is located on the outer side of the cell membrane

The trityrosine residues in resilin can be formed by a reaction between a tyrosine radical and a dityrosine radical

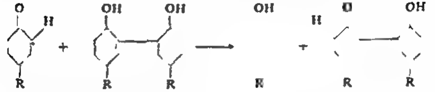


The dityrosine radical involved in the process may be incorporated in either one or two peptide chains In the following I shall assume that it connects two chains and that the formation of a trityrosine residue results in the linking together of three chains however even if all three tyrosine residues are located in the same chain the arguments will not be affected

The pathway for the formation of dityrosine radicals depends on whether the oxidizing enzyme performs its action during or after the secretion If the material is still available for enzymatic oxidation after it has been secreted

the formation of dityrosine radicals can be a straightforward oxidation of the dityrosine residues by means of the enzyme provided that the specificity of the enzyme allows oxidation of biphenolic compounds

If proresilin is oxidized enzymatically only during the secretion dityrosine radicals cannot be formed in this way because the dityrosine residues must be formed after the secretion In this case other ways of oxidation must be possible for instance by means of tyrosine radicals



The dityrosine radical formed in this way could then react with another tyrosine radical resulting in the formation of a trityrosine residue

In both schemes the precursors are radicals of tyrosine and dityrosine. This implies that a proteinbound tyrosine radical should be able to get into close contact with a dityrosine radical linked into two protein chains This contact is presumably difficult if the neighbouring amino acid residues carry bulky side chains and should occur easier if the tyrosine and dityrosine residues are surrounded by glycine residues Such a distribution of glycine residues is feasible since more than one third of the residues in resilin are glycyl The amino acid sequence around the tyrosine residues in proresilin may also determine which tyrosine residues are going to be oxidized by the enzyme and consequently participate in the formation of cross links It would be interesting to determine the amino acid sequence surrounding the tyrosine and the di and trityrosine residues in resilin to see whether glycine occurs more often near di and trityrosine residues than near the unreacted tyrosine residues

The discussion on the biosynthesis of resilin has only concentrated on the formation of the cross-links but other problems are present which are of at least equal interest For instance why is the protein devoid of secondary structure? This question presumably also applies to proresilin Another problem concerns the factors which govern the regular rhythmic deposition of resilin and chitin lamellae Apparently there must be some mechanism which switches the deposition over from chitin to resilin and *vice versa* The solution of these problems demands much work to be done but it is now becoming possible to ask specific questions which can be tested experimentally

8 Comparison with some other types of cross-linked proteins

Although cross links are present in many proteins their identity has only been established with certainty in a few cases It is therefore too early to discuss the connection between the type of cross link and the structure and function of the proteins in which it occur However it can be of interest to compare resilin with a few other cross linked systems in order to see if any common characteristics are present The two other systems chosen for comparison are elastin the protein with rubberlike elasticity occurring in vertebrates and the hard sclerotized cuticle in insects which is produced by epidermal cells similar to those producing resilin

RESILIN

As discussed in the foregoing chapters this protein consists of a huge isotropic three dimensional molecular network of randomly coiled cross linked polypeptide chains It is deposited in structures where elasticity is essential for the proper function In some cases where the structure is exposed only to stretching no material other than resilin is present but structures which are exposed to bending are usually composed of alternating lamellae of chitin and resilin

The deposition of resilin may last a few days (dragonfly tendon) or a few weeks (locust ligaments) and it is performed by a single layer of epidermal cells The peptide chains become cross linked just outside the cells with the result that the structures only grow by one sided surface apposition The regular array of alternating layers of chitin and resilin as well as the final form of the ligaments are apparently determined solely by the epidermal cells

Resilin when first deposited appears to have no turnover in the adult insects This may be due to the absence of pore canals from these parts of the cuticle However in the nymphal stages there must be some turnover of resilin The nymphs are unable to fly have no wing hinges and no prealar arms but resilin is present in the clypeo labral spring in the tarsal pads and in the

margins of the abdominal tergites Part of this resilin is dissolved by the moulting fluid immediately before a moult in the same way as the other cuticular structures We do not know anything about the fate of the cross links after this digestion but they are presumably resorbed by the underlying epidermal cells together with the other amino acids liberated It would be interesting to know something about their further metabolism in the animals

ELASTIN

This protein is present as elastic fibres in vertebrate connective tissue the aortic wall and the *ligamentum nuchae* being especially rich samples It always occurs together with collagen and mucopolysaccharides, and a drastic treatment of the tissues is necessary to obtain pure samples of elastin This purification may easily result in some degradation of the product

The properties of elastin have been reviewed by *Partridge* (1962) The protein appears to be similar to resilin in many respects Elastin fibres also show rubberlike elasticity (*Hoeve* and *Flory* 1958) they are isotropic when unstrained and they swell in protein solvents but are completely insoluble in all solvents which do not degrade proteins There is also some resemblance between elastin and resilin with respect to amino acid composition both have a high content of glycine, a moderately high content of proline but elastin differs from resilin in being very poor in amino acids with polar side chains

Both proteins are fluorescent in ultra violet light and peptides containing cross links have been purified from partial hydrolysates of elastin (*Partridge* 1962) From these peptides the two amino acids desmosine (A) and isodesmosine (B), have been isolated (*Partridge Elsden* and *Thomas* 1963)

The structures of the desmosines indicate that they can be formed from four lysine residues and tracer experiments with radioactive lysine indicate that this amino acid is the precursor (*Partridge et al* 1964 *Miller Martin* and *Piez* 1964) The experiments of *Miller Martin* and *Piez* (1964) indicate that there is an appreciable time lag between the incorporation of lysine in the elastin and the transformation of lysine into cross links For one day they grew isolated aortas from chicken embryos in the presence of labelled lysine and thereafter in the presence of unlabelled lysine for different periods of time The elastin was isolated, hydrolysed and the radioactivity of lysine and desmosines was determined The results show that whereas labelled lysine is rapidly incorporated in elastin it takes almost two weeks to transform the incorporated lysine into desmosines The process involved in the formation of desmosine cross-links in elastin therefore appears to be much slower than the process involved in the cross linking in resilin

The measurements of *Miller Martin* and *Pie* (1964) indicate an interesting feature not commented upon by the authors In aortas analyzed after one day s growth in the presence of labelled lysine the elastin contained much labelled lysine and only insignificant amounts of labelled desmosine The labelled lysine must be located in the most recently synthesized elastin which appears to be effectively cross-linked to the older elastin since it can withstand the drastic purification procedure (extraction with 0 1 M NaOH at 98° C for one hour) This indicates that besides the slowly formed desmosines elastin may also contain some type of rapidly formed cross-links of unknown nature

Furthermore *Miller Martin* and *Pie* 1964) found that the ratio of lysine to desmosines in elastin changes during the development of the chickens whereas the sum of lysine and quarter-desmosines remains constant The results indicate that the transformation of lysine residues into cross-links is a slow process which may last weeks or months During early development the formation of new elastin is rapid compared with the velocity of the cross-linking process resulting in a small relative amount of cross links The synthesis of a new elastin is very slow in older animals and therefore their elastin will be fully cross-linked

Copper deficiency in chicken and swine results in high mortality associated with rupture of the aorta and the coronary arteries (*O Dell et al* 1961 *Coulson* and *Carnes* 1963) The amount of elastin in these animals is lower than normal and it appears to be less cross-linked since it swells more and is more easily brought into solution than is normal elastin (*Starcher Hill* and *Matrone* 1964) Amino acid analyses have shown that the lysine content is about three times higher in elastin from the copper deficient animals than in normal elastin (*Starcher Hill* and *Matrone* 1964) A high amount of soluble proteins can be extracted from aortas from the deficient animals and this soluble protein is similar to elastin with respect to the proline hydroxyproline ratio (*Weissman Shields* and *Carnes* 1963) It is possible that this soluble protein represents proelastin the non-cross linked precursor

The role of copper in the cross-linking process has not been clarified but it has been suggested that a copper-containing enzyme is involved in an oxidative process which makes the lysine residues in the proelastin reactive (*Partridge et al* 1964) This idea has no experimental support as yet but the problems are under active investigation in several laboratories

In a protein with ideal rubberlike elasticity no stable secondary structure must be present under the conditions prevailing in the organism the chains have to be thermally agitated and in a randomly coiled configuration and stable cross-links must be present between them The elasticity becomes most

perfect when the distance along the chains between two cross links is almost the same throughout the material In extra-cellular proteins the cross-linking process must occur outside the cells so that material secreted from several cells can be linked together It is therefore assumed that a soluble non-cross linked precursor is first secreted and thereafter cross linked both in the case of resilin and elastin As the peptide chains have to be randomly coiled after the cross linking they must also have been in this state before meaning that the absence of a stable secondary structure must be inherent in the peptide chains and not due to the formation of cross links between them It has been suggested that the distribution of proline residues in the peptide chains could be the respon sible factor both in resilin and in elastin (*Andersen* and *Weis Fogh* 1964) However this is a problem where more work is strongly needed before final conclusions can be drawn

It was mentioned above that the most perfect rubberlike elasticity is to be expected when the distance between two neighbouring cross links is almost constant This can be obtained if the non-cross linked precursor has a primary structure with equal spacing between those amino acid residues from which the cross links are going to be formed Since only half of the tyrosine residues in proresilin take part in the process it is possible that a certain amino acid sequence is needed around a tyrosine residue in order to enable it to parti cipate in the cross linking (cf p 63) In this way it would be possible to ensure a given distance between the cross-links although the pairing of the tyrosine radicals occurs completely at random

In the case of elastin where four lysine residues are involved in the forma tion of a single cross link it has been suggested (*Partridge et al* 1964) th[illegible] three of them are located very closely together in the same peptide chain [illegible] that after oxidation they react with each other and with a fourth [illegible] fied lysine residue occuring in another peptide chain This will resul[illegible] formation of a cross link between only two peptide chains althou[illegible] sidues are involved The idea has no experimental foundation [illegible] tractive since it does not demand that four lysine residues locat[illegible] dual peptide chains have to come together in order to establi[illegible] As soon as the precursor of elastin has become isolated in a pur[illegible] be possible to demonstrate whether such a cluster of three ly[illegible] tually occurs

Whereas the resilin producing cells occur in a single con[illegible] result that uni directional growth takes place the cells [illegible] sible for the production of elastin in vertebrates are sc[illegible] connective tissues and seem to be identical with the [illegible] and the mucopolysaccharides of the ground substa[illegible]

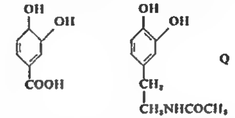
ment of the animals the elastin producing tissues grow continuously, and their form and mechanical properties must be adjusted according to the needs of the animal It is therefore important that the final cross linking of elastin is not instantaneous since the soluble precursors formed by the individual cells must have time to become mixed before the cross linking and there must be sufficient time for the surrounding tissues to influence the form of the elastic network resulting from the cross linking

SCLEROTIZED CUTICLE

The mechanical properties of the major parts of the insect cuticle differ strongly from those of the resilin containing parts although all parts consist mainly of chitin and protein (*Jensen* and *Weis Fogh* 1962) The non rubbery cuticle can roughly be divided into two types a soft but inextensible type present for instance in the intersegmental cuticle, and a hard and inextensible type present in the sclerotized areas Several aspects of the chemistry and physiology of cuticle have been much investigated and reviewed (*Cottrell* 1964, *Dennell* 1958 *Hackman* 1959 *Pryor* 1962 *Richards* 1951 1958 *Wigglesworth* 1948 1957) but many problems are still unsolved and some aspects have been almost completely neglected It is therefore only possible to offer a preliminary picture of the formation of insect cuticle but the general principles appear to be sufficiently well established to allow a comparison between the formation of solid and rubberlike cuticle The processes involved in the sclerotization of the solid cuticle can be illustrated by the processes which take place during the hardening of the cockroach ootheca although this is a non cuticular example (*Pryor* 1940 a b)

The eggs laid by cockroaches are surrounded by a protein matrix which is flaccid and uncoloured when secreted and rapidly becomes hard dark and unusually resistant towards enzymes and chemical reagents thus forming a protection for the eggs This hardening is due to enzymatic oxidation of protocatechuic acid (3 4 dihydroxybenzoic acid (P)) to the corresponding quinone The quinone reacts with free amino groups in the protein each quinone molecule being able to react with at least two amino groups In this way the protein becomes randomly cross linked and the hardness of the final product depends on the degree of cross linking determined by the amount of quinone formed and the number of free amino groups in the protein During the hardening the number of lysine residues decreases from 9 7 per cent to 3 6 per cent confirming that this amino acid is involved in the process (*Hackman* and *Goldberg* 1963) The protein to become tanned is secreted from the left colleterial gland in the cockroach and this gland also secretes a glucoside of protocatechuic acid and an oxidase capable of oxidizing free protocatechuic

acid but not the glucoside At the same time the right colleterial gland secretes a glucosidase able to hydrolyse the glucoside thus setting the protocatechuic acid free (*Brunet* and *Kent* 1955 *Kent* and *Brunet* 1959) Therefore the tanning reaction can first start when the two secretions are mixed

P  Q

A similar, although not identical tanning occurs during the formation of the blowfly puparium In this case the soft light coloured larval cuticle is transformed into a hard dark puparium Just before the formation of the puparium the larvae are rich in *N* acetyl dopamine (Q), but this compound disappears abruptly at the time of pupation (*Karlson Sekeris* and *Sekeri* 1962) At the same time an enzyme appears which is able to oxidize acetyl dopamine to the corresponding quinone this enzyme was present beforehand as an inactive proenzyme (*Schweiger* and *Karlson* 1962) When the larvae are injected with radioactive tyrosine or dihydroxyphenylalanine before the pupation most of the activity becomes incorporated in the puparium (*Karlson* 1960) and autoradiography of such labelled puparia shows that the labelling appears mainly in the sclerotized parts of the puparium (*Ammon* and *Karlson* 1964) These results indicate that the reactions occurring during the puparium formation are similar to the processes occurring during the tanning of the cockroach ootheca a main difference being the nature of the diphenol which is oxidized to a quinone

It is generally assumed that the process involved in the sclerotization of insect cuticle follows a similar scheme but the individual steps involved have not yet been elucidated in detail It is probable that the reactions involved in the sclerotization are not completely identical in all insect species, and it is even possible that differences may exist between the different parts of the sclerotized cuticle in a given insect Differences could thus be present between the areas of the cuticle sclerotized before emergence and those sclerotized afterwards (*Cottrell* 1964)

The formation of the new cuticle generally starts a few days before the insect is going to moult The new cuticle is deposited beneath the old one which at the same time becomes partly digested by the moulting fluid secreted by the epidermal cells The new cuticle consists of an outer layer called epicuticle which is reported to contain lipids and proteins but no chitin (*Ri*

chards 1958 *Wigglesworth* 1957) and beneath this layer the exocuticle is deposited This part of the cuticle consists mainly of chitin and soluble protein(s) and is soft but without rubberlike elasticity After the insect has emerged from the remnants of the old cuticle it distends itself by swallowing air (*Cottrell* 1964) and the final form of the insect depends on the local properties of the new cuticle which are determined by the underlaying layer of epidermal cells Immediately after the insect has obtained its final form, parts of the soft cuticle are tanned and become hard and in some places it also becomes darkly coloured This tanning is apparently performed by quinones reacting with the originally soluble proteins present in the cuticle but there is still some controversy about the identity of the quinones involved However, it need not be the same quinones which are involved in the tanning in all insect species In the case of *Schistocerca gregaria* the quinone is apparently obtained from *N* acetyl-dopamine by enzymatic oxidation (*Karlson* and *Sekeris* 1962) If radioactive tyrosine or acetyl dopamine is injected during the period 15–70 hours before a moult about 50 per cent of the radioactivity becomes incorporated in the cuticle during the first few hours after emergence Tyrosine injected at an earlier stage is mainly metabolized by another route and is not utilized for tanning (*Karlson* and *Schlossberger Raecke* 1962 *Schlossberger Raecke* and *Karlson* 1964)

The cuticle of locusts grows in thickness for several weeks after the emergence (*Jensen* and *Weis Fogh* 1962) Almost no work has been done to elucidate the state of the proteins in this endocuticle whether they are soluble linked to the chitin by means of covalent bonds as suggested by *Hackman* (1959) or whether they are cross-linked Cross linking is indicated by the fact that the elastic modulus of exocuticle is almost identical with that of samples in which endocuticle is more abundant (*Jensen* and *Weis Fogh* 1962)

According to the examples discussed here cross linking of proteins by means of quinones occurs in those instances where a preformed protein rapidly has to become hard and insoluble In the case of the ootheca and the puparium the primary function of these sclerotized proteins must be one of protection The insect cuticle is a much more elaborate structure where the different parts are specialized according to their functions and it can therefore be expected that different processes will be involved in the formation of these parts So far details are mainly known for the rubberlike resilin-containing cuticle and for the solid quinone tanned cuticle and in these cases two completely different reactions have been used to obtain the final products In resilin-containing cuticle a continuous cross linking occurs by means of pairing of free radicals formed by oxidation of tyrosine residues during the deposition of the protein In quinone tanned cuticle a bulk tanning of an already deposited protein occurs by means of a small reactive molecule reacting with

free amino groups It is possible that some types of cuticle are cross linked by the combined action of these two methods a rubberlike protein may be quinone tanned to give it a greater stiffness, and a small amount of cross links may be present in some proteins in insect cuticle before sclerotization (*Jensen* and *Weis Fogh* 1962) This could serve as means to immobilize the soluble proteins since they would be captured in the network of the cross linked proteins

We have tried artificial quinone tanning of resilin-containing ligaments by keeping them in a saturated solution of *p* benzoquinone for several hours (*Jensen* and *Weis Fogh* 1962 *Andersen* and *Weis Fogh* 1964) By this treatment the ligaments became dark brown but they were still rubberlike the only difference being a slight difference in the swelling behaviour Such tanned elastic structures have not been noted in insects, but they resemble a part of the hinge ligament in the scallop *Pecten* The central inner part of this ligament consists of a dark brown elastic gelatinous mass and almost no hysteresis was observed when the ligaments were alternatively strained and relaxed (*Trueman* 1953 a b) Little is known about the possible cross links *Trueman* (1953 b) suggesting that the proteins are quinone tanned

In order to investigate the possibility that cross-links like those in resilin are present in small amounts in the solid cuticle before it becomes sclerotized I have looked for di and trityrosine both in fully hardened and completely untanned samples Samples of untanned cuticle were obtained from locusts just about to emerge The presence of di and trityrosine could not be demonstrated in any of these types of cuticle and the solubility of the proteins in the untanned cuticle indicates that cross-links are not present at this stage However hydrolysates of samples of fully sclerotized cuticle consisting of both exo- and endocuticle contain large amounts of aromatic compounds making the detection of small amounts of di and trityrosine difficult

We have not found any striking similarities between resilin and ordinary solid cuticle However, it is tempting to assume that some sort of similarity exists between these two types The transition zone between the two types of cuticle is always sharp but no differences have been observed in the cells responsible for their deposition In some instances a given group of cells must have started first by making exocuticle then endocuticle and then switched over to the deposition of resilin (Fig 33 in *Andersen* and *Weis Fogh* 1964) This shows that the ability to produce all these types of cuticle can be inherent in a single cell The possibility was considered that the proteins involved could have nearly the same composition and that only the cross linking processes were different However the amino acid composition of protein fractions from different types of locust cuticle differed strikingly from that of resilin (*Andersen* unpublished)

Summary

A list is given of the different types of covalent cross links which have been demonstrated or suggested to occur in proteins. It includes disulphide groups, ester and amide groups, the desmosines present in elastin, and quinone mediated cross-links in sclerotized proteins. The biphenolic cross links in resilin described in this work are also included.

Some of the general properties of resilin are described. The swelling behaviour as well as the rubberlike elasticity show that stable covalent cross links are present in this protein. The cross links cannot be identical with any of the types of cross links described for other proteins.

A detailed description is given of the methods which have been found of value in isolating and purifying two fluorescent compounds (Compound I and II) present in hydrolysates of resilin. Chromatographically pure, salt free samples can be obtained of both compounds. A third fluorescent compound (Compound III) which is present in very small amounts has not been obtained in sufficient quantity to be characterized in detail as yet.

According to colour reactions on paper chromatograms Compounds I and II are α amino acids containing a phenolic group. The phenolic group appears to be remarkably acid as it ionizes about pH 6–7. The combined results from titrations, quantitative colour reactions and partial dinitrophenylation of amino groups demonstrate that Compound II contains two carboxylic groups, two α amino groups and a phenolic group ionizing near pH 7, and that Compound I contains three carboxylic and three α amino groups and a phenolic group ionizing near pH 6. The molar absorption coefficient is 5400 for *Compound II (283.5 mμ) and about 8000 for Compound I (286 mμ).*

The ultra violet absorption spectra, the fluorescence activation and emission spectra, the pH dependence of the absorption and of the fluorescence and the formation of complexes with boric acid indicate that Compound II contains the *o,o'* biphenolic structure, and that Compound I contains the corresponding terphenolic structure. Compounds identical with Compounds I and II are formed when tyrosine is enzymatically peroxidized. Since the main product of

this reaction is dityrosine (*Gross* and *Sizer* 1955, 1959) it is concluded that Compound II is identical with dityrosine (E) and that Compound I is identical with trityrosine (F). The structure tetratyrosine (O) is suggested for Compound III, although the evidence for this structure is not entirely conclusive.

The formation of biphenolic compounds from monophenols either by enzymatic or non-enzymatic oxidations proceeds generally *via* the pairing of free radicals, and it is assumed that this mechanism also applies to the formation of di- and trityrosine in resilin.

Evidence is presented that di- and trityrosine are the actual cross links in resilin. Apparently the amino and carboxylic groups of these compounds are engaged in peptide linkages in the protein. The evidence is not complete but it is supported by the finding that the degree of cross linking in resilin calculated from the measurements of the elasticity corresponds closely to the degree of cross linking calculated from the amounts of di- and trityrosine found in resilin.

Resilin thus appears to consist of a huge molecular network of randomly coiled peptide chains linked together by means of di- and trityrosine residues. Experiments are described where dissolved silk fibroin is cross linked by means of horseradish peroxidase and hydrogen peroxide. This reaction leads to the formation of both di- and trityrosine residues in the fibroin and the end product of the reaction is an insoluble gel having many properties in common with resilin.

A hypothesis for the biosynthesis of resilin is discussed. This hypothesis was originally proposed by *Andersen* and *Weis-Fogh* (1964) before the cross links had become identified, and this new information is now incorporated in the hypothesis. The formation of resilin is thought to proceed *via* a soluble precursor, proresilin, identical with the non-cross linked polypeptide chains in resilin. The precursor is suggested to be secreted from the cells and to be oxidized enzymatically during or immediately after the secretion. During this oxidation free radicals are formed from some of the tyrosine residues, and a dityrosine cross link will then form spontaneously when a tyrosine radical meets a corresponding radical either on the already cross-linked resilin or on a still non-cross linked chain. In this way the deposit of resilin will grow continuously as long as the cells continue to secrete and activate the precursor.

This way of forming a cross linked protein is finally compared with two other cross linked systems, elastin in vertebrates, and sclerotins in insects. Different cross linking processes are involved in these three cases, and the processes are discussed in relation to the properties of the structures in which they occur.

Dansk resumé

Indledningen gennemgår kort de forskellige typer kovalente tværbindinger i proteiner. Denne liste indeholder både tværbindinger, hvis tilstedeværelse og struktur er fastslået med rimelig sikkerhed, og også en del eksempler på proteiner, hvor det må antages, at kovalente tværbindinger er til stede, men hvor de endnu ikke er blevet identificeret. Tværbindinger i proteiner kan have så varierende strukturer som disulfidgruppen i cystin, ester- og amidgrupper, desmosinerne i elastin og tværbindinger dannet mellem kinoner og frie aminogrupper, som det er tilfældet i sklerotiserede proteiner. En ny type tværbindinger kan nu føjes til denne liste, idet tværbindingerne i resilin er identificeret som di- og trityrosin som beskrevet i dette arbejde.

Kapitel to indeholder en beskrivelse af nogle af resilinets egenskaber, idet hovedvægten er lagt på de egenskaber (kvældningsforhold og elasticitet), som førte til den antagelse, at resilin indeholder kovalente tværbindinger. Disse tværbindinger kan ikke være identiske med nogle af de typer, som er beskrevet for andre proteiner.

Derefter gives en detaljeret beskrivelse af de metoder, som er fundet brugelige til at isolere og oprense to fluorescerende stoffer (Compound I og II), som er til stede i resilinhydrolysater. Med de beskrevne metoder kan man få begge de fluorescerende stoffer saltfrie og i kromatografisk ren tilstand. En tredje fluorescerende komponent (Compound III) er kun til stede i meget lille mængde, og det har derfor ikke været muligt at gennemføre meget arbejde med denne komponent.

På papirkromatogrammer giver de to fluorescerende stoffer farvereaktioner, der svarer til, at de er α-aminosyrer, og at de indeholder en phenolgruppe. Denne phenolgruppe er en betydeligt stærkere syre (pK 6–7) end normale phenolgrupper (pK ca. 10). Ved hjælp af titrering, kvantitativ ninhydrinreaktion og delvis dinitrofenylering af aminogrupper har det kunnet vises, at Compound II indeholder to carboxylgrupper, to α-aminogrupper og en phenolgruppe, der dissocierer ved pH 7. Compound I indeholder tre carboxylgrupper, tre α-aminogrupper og en phenolgruppe, der dissocierer ved pH 6.

Den molære absorptionskoefficient er for Compound II fundet til 5100 ved 283.5 mμ og for Compound I til ca. 8000 ved 286 mμ.

De to fluorescerende stoffer er blevet sammenlignet med forskellige model stoffer med hensyn til absorptionsspektre aktiverings- og emissionsspektre for fluorescensen pH afhængigheden af både absorption og fluorescens og evnen til at danne komplekser med borsyre. Denne sammenligning viser at Compound II indeholder o o biphenolstrukturen og at Compound I er en tilsvarende terphenol. Ved enzymatisk peroxidering af tyrosin kan der dannes fluorescerende stoffer, der er identiske med dem, der er isoleret fra resilin. Det er blevet vist, at der ved denne peroxidering af tyrosin dannes dityrosin (*Gross* og *Sizer* 1955, 1959). Alle de opnåede resultater svarer nøje til, at Compound II er dityrosin (E), og at Compound I er trityrosin (F). Compound III foreslås at være tetratyrosin (O), selv om grundlaget for denne slutning endnu er utilstrækkeligt.

Den oxidative dannelse af biphenoler fra monophenoler foregår over frie radikaler. Dette gælder, hvadenten oxideringen er enzymatisk eller ikke, og det foreslås at denne mekanisme også gælder ved dannelsen af di- og trityrosin i resilin.

Det er forsøgt fastslået hvorledes di- og trityrosin er bundet i resilin, og de opnåede resultater viser at dette sandsynligvis må være gennem normale peptidbindinger, således at alle amino- og syregrupper i begge stoffer er involveret i peptidbinding. På denne måde kan begge aminosyrer fungere som tværbindinger mellem peptidkæderne. Der er god overensstemmelse mellem den tværbindingsgrad der kan beregnes ud fra de fysiske målinger og den der kan beregnes ud fra mængden af di- og trityrosin.

Resilin må således bestå af et uhyre stort molekyleret netværk opbygget af tilfældigt snoede peptidkæder der holdes sammen ved hjælp af di- og trityrosinrester. Forsøg beskrives hvor opløst silkefibroin tværbindes ved hjælp af hydrogen peroxid og en peroxidase. Denne reaktion fører til dannelsen af både di- og trityrosinrester, og som slutprodukt af reaktionen fås en uopløselig gel af silkefibroin denne gel ligner resilin i mange henseender.

Biosyntesen af resilin diskuteres på grundlag af en hypotese fremsat af *Andersen* og *Weis Fogh* (1964). Denne hypotese blev udarbejdet inden tværbindingernes struktur var klarlagt og det diskuteres nu hvorledes hypotesen kan udbygges ved hjælp af denne viden. Resilin tænkes dannet ud fra et opløseligt protein proresilin identisk med de ikke tværbundne peptidkæder i resilin. Dette proresilin syntetiseres i cellerne og secerneres fra disse. Under eller umiddelbart efter denne sekretion iltes nogle af tyrosinresterne enzymatisk til frie radikaler og to sådanne radikaler vil spontant danne en dityrosinrest hvis de mødes. Denne dannelse af tværbindinger vil finde sted både mellem de secer

nerede proresilinmolekyler indbyrdes og mellem disse og det allerede tværbundne resilin. På denne måde kan aflejringen af resilin fortsætte, så længe cellerne fortsætter med at secernere og aktivere proresilin.

Til sidst sammenlignes dette system med to andre systemer, hvor der også forekommer kovalente tværbindinger: elastin hos hvirveldyr og sklerotin hos insekter. Disse tre systemer tværbindes på forskellig måde, og deres ligheder og uligheder diskuteres.

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Electrical Properties and Fine Structure of the Ampullary Canals of Lorenzini

By

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Preface

This paper consists of two parts. Part I considers the ampullary canals of Lorenzini as electrical conductors and gives a quantitative description of their cable properties. Part II describes the histology of the organs by light and electron microscopy; it seeks primarily to correlate structure with electrical properties but treats as well other morphological features that are considered important for an understanding of the function of the ampullary canal.

The title and text employ the designation 'ampullary canals of Lorenzini' rather than the usual 'ampullae of Lorenzini'. It was Boll (1868) who proposed *Lorenzini'schen Ampullen* to supplant the earlier *Schleimröhren* (mucus tube), citing as authority the use of *Ampullen* by Müller (1851) and Leydig (1852). But the latter had distinguished between *Ampullen* and *Rohren*, and Todaro (1870) rejected Boll's proposal, observing "*che l'ampolla costituisce solamente una parte del tubo ... il nome di tubo debba essere rispettato*". Indeed Lorenzini (1678) had himself paid as much attention to the canals as to their terminal expansion. The Oxford Dictionary defines ampulla as the 'dilated end of vessel, canal, duct in an animal', and ampulla of Lorenzini in current usage generally means only the dilated end, although it has been employed for canal and ampulla together. Dijkgraaf (1963) circumvented the ambiguity with 'organs of Lorenzini', which has the additional merit of brevity but is not especially descriptive. A more specific name is that used by Ewart & Mitchell (1892): 'ampullary canals'. If this is increased to 'ampullary canals of Lorenzini', no doubt should arise as to what organs are intended. In this paper, then, 'ampullary canal of Lorenzini' or 'ampullary canal' denotes the entire structure from skin pore to proximal end; 'ampulla' means only the bulb at that end; and 'canal' means the duct connecting the ampulla to the skin surface.

I am pleased to express my indebtedness to members of the several institutions where this investigation was pursued.

At the Nobel Institute for Neurophysiology: Professor Ragnar Granit, its director, for his liberal hospitality and unsparing support; Professor

Bernhard Frankenhaeuser, for kindly providing laboratory space and apparatus and for much valuable advice and discussion, Laborator Curt von Euler, for friendly encouragement, Dr Barry Lindley, for critically examining the first draft of Part I, Ing Erik Persson, for invaluable help in equipping the laboratory at Kristineberg, Fru Eva Reigo, for skilful execution of the graphic illustrations, and Fröken Gunvor Larsson, for painstaking preparation of the typescript and for other much appreciated assistance.

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At the Kristineberg Zoological Station, Laborator Bertil Swedmark, its director, for placing working facilities at my disposal and his staff for their considerable efforts in collecting material.

At the Computations Division of the Karolinska Institute, Ing Bruno Lundberg, its director, for programming equation (1 8) and for freely providing time for computation.

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B W

Stockholm
November 1965

I Electrical Properties of the Ampullary Canals of Lorenzini in *Raia*

INTRODUCTION

When Lorenzini (1678) described the ampullary canals that now bear his name, he thought the ampullae were glands and, at first sight, that the long canals served to distribute their gelatinous secretion over the surface of the fish. After closer inspection, however, he commented that "... the walls of the canals, which, being much thicker than what is appropriate for a simple duct, make us suspect that they are intended for another, more hidden function, since nature never acts casually, nor multiplies entities without necessity."*

To-day the ampullae are known to be organs of sense, not secretion, and it is reasonable to expect that the canals serve to conduct stimuli to them. But attempts to identify the biologically adequate stimulus have been repeatedly embarrassed by the apparent inappropriateness of the canal in structure and distribution for discharging this function. Afferent responses from the ampullae have been demonstrated electrophysiologically to very small temperature changes (Sand, 1938), to weak tactile disturbances at the ends of the canals (Murray, 1960 *a*), to pressure differences across the ampullary wall (Murray, 1957; Loewenstein, 1960), and to alteration in CO_2 concentration (Hensel, 1957); yet the reception of none of these stimuli would appear to require or be served by the interposition of a long, flexible, jelly-filled canal between the sensory epithelium and the sea-water environment.

More promising is the observation by Murray (1960 *b*, 1962) that the ampullae are responsive to weak electrical stimuli applied, for example, as voltage gradients in the ambient sea water. Electrical sensitivity was also reported by Loewenstein & Ishiko (1962). As this modality is not so many

* "... pareti de' canali, le quali essendo di gran lunga più grosse di quello che si conviene ad un semplice condotto, fanno dubitare d'essere destinate a qualche altro uso più nascosto, giacchè la natura non opera mai a caso, nè multiplica gli enti senza necessità."

festly incompatible with the anatomical features of the canals the possibility that the ampullae are electroreceptors as Lissmann (1958) had already suggested has been given serious consideration which Dijkgraaf (1963) reviews But while it has been inferred from the comparatively high conductivity of the jelly that preferential channelling of electric currents occurs in the canals (Machin 1962) no adequate evidence has hitherto been presented that they do indeed subserve this function

As Kelvin (Thomson 1856) showed for the submarine cable the passive spread of potential or current along a core conductor is determined not only by the resistance of its core but also by its insulation and the nature of its termination the first two factors receiving expression in a space constant or characteristic distance λ The resistivity of the jelly expressed from the canals is 25 Ω cm (Murray & Potts 1961) In the absence of other information a reasonable assumption is that the mural resistance of the canal is the same as that of the skin from which the canal arises The only published measurements of skin resistance in an elasmobranch *Torpedo* (Bennett 1961) permit at most a value of 2 500 Ω cm² From these data and the canal diameter of 0 4 mm given by Murray (1962), λ may be estimated to be one cm Canal length in the same preparation was 0 2 to 2 0 cm (Murray 1960*a* Fig 2) which cannot be assumed infinite compared to λ If the ampullary end of the canal is assumed to be insulated the steady state attenuation of a stimulus of unit potential applied at the pore will be least for a given value of λ and may be estimated with the first term of eqn (1 10) given below (cf Weidmann 1952) In the shortest ampullary canal the decline would be negligible while in the longest the stimulus would be down to about one quarter at the ampulla As Murray (1962) measured the threshold for a 10 % change in the afferent discharge frequency any attenuation in the longer canal may have been observed as an elevation in threshold which did show a more than tenfold variation

The observations of Loewenstein & Ishiko (1962) were made on ampullary canals about one cm long and are equally inconclusive in this regard No special adaptation of the canals as electrical conductors can therefore be assumed from the published results What is required is evidence that serious attenuation would not occur even in the metre long canals that are found in a skate of 2 m span Their calibre is about 2 mm yet even if they were insulated by a myelin sheath (0 1 MΩ cm) λ in them would be less than 15 cm by the above argument

If the canal is to provide reasonably lossless conduction of electrical stimuli it must either possess insulation of an extraordinary kind or else

like a nerve discharge a propagated response. T... investigated in the following way

1. Theoretical equations were derived for the pas... pullary canal to the application of a voltage ... constants: mural resistance, mural capacity and ...
2. The response of the canal to a rectangular vo... experimentally in a series of ampullary canal...
3. The three constants were measured in other ca...
4. Numerical solutions of the theoretical equa... inserting the measured values of the con... were fitted to the experimentally recorded re...

THEORY

Consider the ampullary canal as a ... conducting core and a thin surface wal... mersed in a large volume of conducting ... potential everywhere

If

x is distance along the canal in cm
t is time in sec
V is the transmural potential in volts
I is the longitudinal current in ampe...
a is the radius of the canal in cm
l is the length of the canal in cm
R is the resistivity of the core in Ω c...
R_m is the mural resistance $\times$ unit area
C_m is the mural capacity per unit area
r is the core resistance per unit lengt...
r_m is the mural resistance $\times$ unit lengt...
c_m is the capacity per unit length in ...
$\lambda = \sqrt{(r_m/r)} = \sqrt{(aR_m/2R)}$ and is the ...
$\tau_m = r_m c_m = R_m C_m$ and is the mural ti...

then the relations satisfied by V and I ...

$$r_i I = \ldots$$

and

$$c \frac{\partial V}{\partial t} + \frac{V}{r_m} = \ldots$$

Eliminating I

$$\lambda^2 \frac{\partial^2 V}{\partial x^2} - \tau_m \frac{\partial V}{\partial t} - V = 0 \tag{1.1}$$

At $x = 0$ the canal is terminated by the ampulla. It is assumed that this end is effectively insulated, whence the boundary condition

$$\frac{\partial V}{\partial x} = 0 \qquad x = 0 \qquad \text{for all } t \tag{1.2}$$

At $x = l$ a rectangular step of voltage E is applied at $t = 0$

$$V = E \qquad x = l \qquad t > 0 \tag{1.3}$$

By the Laplace transformation* the subsidiary equation of (1.1) with zero initial current and potential is

$$\frac{d^2\bar{V}}{dx^2} - q^2\bar{V} = 0 \qquad 0 < x < l \tag{1.4}$$

where
$$q^2 = r\,(pc_m - 1/r_m) \tag{1.5}$$

The boundary conditions corresponding to (1.2) and (1.3) are

$$\frac{d\bar{V}}{dx} = 0 \qquad x = 0$$

and
$$\bar{V} = E/p \qquad x = l$$

The solution of these is

$$\bar{V} = \frac{E \cosh qx}{p \cosh ql} \tag{1.6}$$

Expressing the hyperbolic functions in (1.6) in terms of negative exponentials and expanding in a series by the binomial theorem

$$\bar{V} = \frac{E}{p} \sum_{0}^{\infty} (-1)^n e^{-q[(2n+1)l - x]} + \frac{E}{p} \sum_{0}^{\infty} (-1)^n e^{-q[(2n+1)l + x]} \tag{1.7}$$

The function whose transform is $(1/p)e^{-qy}$, where y is the expression in the square brackets in (1.7), may be obtained from the appropriate Fourier coefficient pair in Campbell & Foster (1931, Table II, No. 5). On substituting the result in (1.7), the solution is

* The Laplace transform $\bar{f}(p)$ of $f(t)$ is defined as

$$\bar{f}(p) = \int_0^\infty e^{-pt} f(t)\,dt$$

where the real part of p is positive and large enough such that the integral converges.

$$V = \frac{E}{2}\sum_{n=0}^{\infty}(-1)^n\left\{e^{-[(2n+1)l-x]/\lambda}\operatorname{erfc}\left[\frac{(2n+1)l-x}{2\lambda\sqrt{T}}-\sqrt{T}\right]+\right.$$

$$\left.+e^{[(2n+1)l-x]/\lambda}\operatorname{erfc}\left[\frac{(2n+1)l-x}{2\lambda\sqrt{T}}+\sqrt{T}\right]\right\}+$$

$$+\frac{E}{2}\sum_{n=0}^{\infty}(-1)^n\left\{e^{-[(2n+1)l+x]/\lambda}\operatorname{erfc}\left[\frac{(2n+1)l+x}{2\lambda\sqrt{T}}-\sqrt{T}\right]+\right.$$

$$\left.+e^{[(2n+1)l+x]/\lambda}\operatorname{erfc}\left[\frac{(2n+1)l+x}{2\lambda\sqrt{T}}+\sqrt{T}\right]\right\} \quad (1$$

where
$$\operatorname{erfc} z = 1-\operatorname{erf} z = \frac{2}{\sqrt{\pi}}\int_z^{\infty}e^{-u^2}\,du$$

and
$$T = t/\tau_m$$

Equation (1.6) may also be inverted by means of the Fourier-Me[llin] theorem, whence

$$V = \frac{E}{2\pi i}\int_{\gamma-i\infty}^{\gamma+i\infty}\frac{e^{pt}\cosh qx}{p\cosh ql}\,dp \qquad \gamma>0 \qquad (1$$

where $i=\sqrt{(-1)}$. By transformation to a closed contour and the use [of] the calculus of residues the line integral in (1.9) may be replaced by [$2\pi i$] times the sum of the residues at its poles. A similar problem is treated [in] Carslaw & Jaeger (1945, § 87). The solution obtained in this way is

$$V = \frac{E\cosh x/\lambda}{\cosh l/\lambda}-\frac{4E}{\pi}\sum_{n=0}^{\infty}\frac{(-1)^n e^{-[1+(2n+1)^2\pi^2\lambda^2/4l^2]t/\tau_m}\cos(2n+1)\pi x/2l}{(2n+1)\{1+[2l/(2n+1)\pi\lambda]^2\}} \quad (1$$

The first term of (1.10) is the steady state solution obtainable dire[ctly] by solving (1.1) with $\partial V/\partial t=0$ and the boundary conditions (1.2) [and] (1.3).

Solutions (1.8) and (1.10) are complimentary, (1.8) converging m[ore] rapidly when $\lambda^2 t/l^2\tau_m<1$ and (1.10) when $\lambda^2 t/l^2\tau_m>1$.

Fig. 1 gives the time course of the response at $x=0$ for several va[lues] of l/λ.

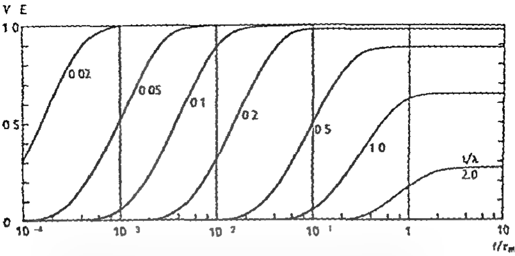

Fig. 1. Theoretical time course of potential at the ampullary end ($x = 0$) of an ampullary canal when a voltage step is applied at the other end ($x = l$), computed with eqn. (1.8) for different values of the ratio l/λ. Abscissa: time as a fraction of the mural time constant, on a logarithmic scale. Ordinate: potential as a fraction of the applied voltage.

When $R_m \to \infty$, $1/R_m \to 0$ and eqn. (1.5) reduces to $q^* = rc_m p$. Solutions of (1.6) are then

$$V = E\sum_{n=0}^{\infty}(-1)^n \operatorname{erfc}\frac{(2n+1)l-x}{2\sqrt{T}} + E\sum_{n=0}^{\infty}(-1)^n \operatorname{erfc}\frac{(2n+1)l+x}{2\sqrt{T}} \tag{1.11}$$

and

$$V = E - \frac{4E}{\pi}\sum_{n=1}^{\infty}\frac{(-1)^n}{(2n-1)}\, e^{-T(2n-1)^2\pi^2/4l^2}\cos\frac{(2n-1)\pi x}{2l} \tag{1.12}$$

where $$T = t/rc_m$$

The steady state solution is the first term of (1.12), i.e. $V = E$.

Equations (1.11) and (1.12) correspond to the response to a voltage step of an ideal submarine cable (zero leakage conductance and zero inductance) that is terminated by an open circuit (Carslaw & Jaeger, 1948, § 64, Ex. 3).

Validity of assumptions

The assumptions that the ampullary canal has a cable-like structure, that the resistances are ohmic and the capacity behaves like a pure dielectric are similar to those made by Hodgkin & Rushton (1946) for a nerve fibre and are considered equally valid here.

The assumption of an insulated end at the ampulla will be justified by showing experimentally that the potential gradient near the ampullary end of the canal is negligibly small.

MATERIAL AND METHODS

Specimens of *Raia radiata* and *R. batis* were trawled or drifted from the Gullmar fjord or Skagerack and kept in running sea water (salinity 32.5–34.0 ‰) at 12° C or less. The experiments were performed on ampullary canals from the hyoid capsule, in particular the long dorsal canals that run tailwards in a bundle to the posterior wing margin. (Ewart & Mitchell, 1892, designated the five bilateral groups of ampullae in the skate as hyoid, superficial ophthalmic, inner buccal, outer buccal and mandibular. This nomenclature has been generally followed although Sand, 1938, and Murray, 1960 *a*, both refer to the hyoid group as hyomandibular.)

The fish was pithed, the great vessels transected in the pericardium to arrest bleeding elsewhere, and the skin reflected over the canals which were then freed in a group from the underlying muscle and removed together with part of the hyoid capsule and nerve. This stage of the preparation was conducted in a cold room at 11°C, under irrigation with Ringer's fluid. Single canals were dissected free in a wax-bottomed trough through which Ringer's at 10° C was circulated.

Stimulation and recording

A transistorised stimulator was used to generate rectangular voltage pulses across a resistance of 560 Ω and to trigger the time base of a double beam cathode ray oscilloscope (Tektronix type 502) which provided DC amplification and display of electrical responses for photographic recording. The Y amplifier for the lower beam of the oscilloscope was fitted with a differential cathode follower input stage. The latter was battery-driven and the grid current in each input valve was reduced to 2×10^{-12} A by adjustment of the anode voltage. Voltage calibration was obtained from the calibrator in the oscilloscope, time calibration from the 50 cycle mains and from a clockwork switch giving 2 c/s.

Measurement of passive spread of potential

The experimental arrangement is depicted in Fig. 2. A single intact ampullary canal more than 10 cm in length was installed in a partitioned Perspex trough filled with Ringer's solution. The distal (cut) end of the

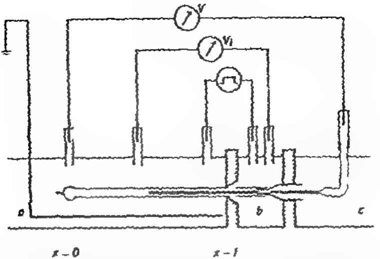

Fig. . Experimental arrangement for recording the response (V_x) of the ampullary canal to a rectangular voltage pulse applied at $x = l$ cm and monitored as V_l. Not to scale. Dimensions: pool *a* 24 cm long, 3 cm wide, 1.5 cm deep; pool *b* 1 × 3 × 1.5 cm; pool *c* 20 × 2 × 1.5 cm. Distance $l = 10$ cm. Ag AgCl electrodes in agar bridges (stippled). Internal pipette filled with 3 M KCl. Further description in text.

canal passed through a hole in the narrow partition separating pools *a* and *b* and was tied over the Ringer filled cannula which traversed the partition between pools *b* and *c*. A thread was tied to a bit of capsular connective tissue left attached to the neck of the ampulla and the canal gently extended into alignment with a mm scale inscribed on the bottom of the trough. A hole was cut in the wall of the canal in pool *b* as close to the partition as possible. Ringer's solution at 10 C was circulated through pool *a* by an air lift.

The internal recording electrode was an L-shaped tube of soda glass, the horizontal limb of which was drawn out to a nearly uniform capillary about 18 cm long and 0.35 mm in diameter. When filled with 3 M KCl it had a d.c. resistance of one MΩ and a capacity of 12 pF/cm length. The electrode was connected to the grid of one input valve by a chlorided silver wire in an agar Ringer bridge and rode together with the valve on the carriage of a micromanipulator used to align the axes of capillary and canal. The capillary was inserted into the lumen of the canal from pool *c* via the cannula and advanced by displacing the trough in a slide fitted with roller bearings against which the trough was spring loaded. The other electrodes were fixed to the trough and moved with it. Small vertical and lateral adjustments were made with the micromanipulator to prevent the capillary from scraping the wall of the canal. A microscope and trans-

mitted light were employed to observe and measure the advance of the tip of the capillary electrode and to measure with the aid of a calibrated eyepiece graticule the canal diameter at 1 cm intervals

Pool *a* was earthed and kept isopotential to within 1 cm of the stimulating site by a chlorided silver wire 2 mm in diameter running the length of the pool this was ascertained with the recording electrode inside the canal by moving the other input electrode during the application of a voltage pulse

The applied stimulus was monitored on the upper beam of the oscilloscope from an independent pair of electrodes in pools *a* and *b* In some experiments pool *c* was earthed to exclude the possibility of stimulating current straying up the surface of the vertical stem of the recording electrode

Measurement of R C_m and R

The ampulla was amputated from an isolated canal and a 6 cm length drawn through holes in the partitions of a Perspex chamber filled with Ringer s solution as shown in Fig 3 Chilled isotonic sucrose solution was flowed around the canal where it passed through the two inner partitions Ringer s solution at 10°C was run into pools *B* *C* and *B* and the over flow drawn off by suction Separate lines and reservoirs for in and out flow were used to maintain electrical isolation between pool *C* and pools *B* and *B* The inflow of Ringer s was brisk enough to prevent the accumulation of sucrose and to hold the temperature near 10 C the latter being controlled by a thermocouple in pool *C*

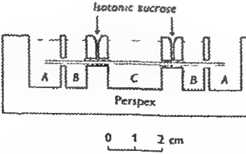

Fig 3 Vertical section through recording chamber used to measure electrical constants of the canal wall Scale as indicated except for boxes given below Isotonic sucrose (stippled) introduced via vertical channels 1 mm in diameter intersecting the horizontal 1 6 mm channels which are traversed by a length of ampullary canal

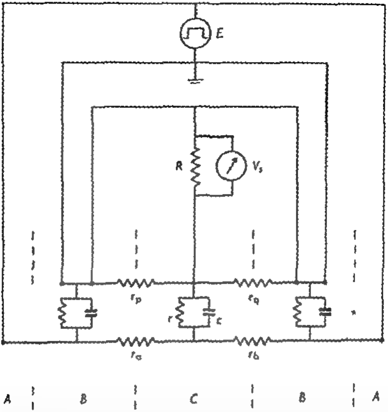

Fig 4 Arrangement of leads and electrodes used with the recording chamber shown in Fig 3 the partitions of which are indicated by vertical dashed lines and the pools by capitals Bold lines in the circuit indicate fluid connexion accentuated intersections indicate electrodes Two additional pairs of electrodes between pools A and B and A and B were used to monitor the applied voltage E For other letter references see text

The arrangement for stimulating and recording is represented by Fig 4 in which

r_a and r_b are the core resistances of the canal halves
r_p and r_q are the resistances of the sucrose gaps
r is the resistance of the canal wall in pool C
c is the capacity of the canal wall in pool C
R is the resistance of a shunt
E is a voltage step applied at time $t = 0$ and
V is the recorded voltage change

The canal wall in pools B and B contributes a resistance and a capacity (represented in Fig 4 by unlettered symbols) in parallel with the branch of the circuit containing r and c and shunted by the stimulator (560 Ω) They are neglected in the analysis to follow because the time taken to

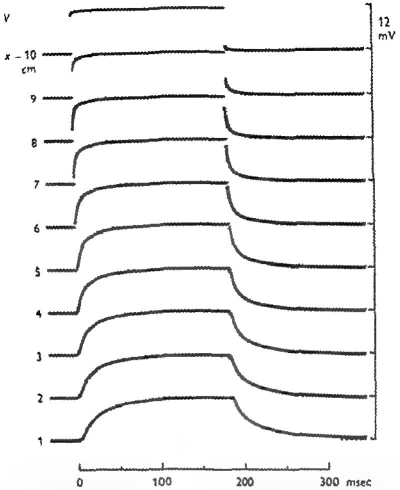

Fig. 6 Experimentally recorded response (V_x) of an isolated ampullary canal at various distances (x) from the ampulla at $x = 0$. Voltage pulse of 12 mV applied at $x = 10$ cm. Each trace is shifted down the voltage axis (ordinate) by 12 mV. *R. radiata* 10 C.

recorded in the arrangement of Fig. 2. The response begins to rise apparently instantaneously in all parts of the canal, the rate of rise gradually diminishing with increase in distance from the site of application of the pulse ($x = 10$ cm). As the applied pulse was constant ($\pm$ 3%) throughout the run, there was almost no decline in steady state response to within one cm of the ampullary end. Steady state amplitude is plotted against distance in Fig. 7 together with the decline predicted by eqn. (1.10) when λ is infinite, 30 cm and 20 cm respectively. It follows from the plot that λ

r_c is given by the instantaneous value of V_s at $t = 0$,

$$V_s(0) = E r_e/(r_c + r_e) \tag{23}$$

r is given by the steady state response

$$V_s(\infty) = E r_e/(r + r_c + r_e) \tag{24}$$

c is found from τ_s

The length of canal in pool C was 2.0 cm and the diameter was measured at 2 mm intervals under 25 × magnification. R_m and C_m were then found from r and c respectively.

Although R_i, the core resistivity, could be derived from r_c, a more direct and accurate measurement was made in the following way. A length of canal was drawn through a greased hole in each of two narrow partitions dividing a long Perspex chamber of small cross section into three pools. The canal was cut off in the outer pools close to the partitions. Isotonic sucrose at 10°C was run continuously into the middle pool and the overflow removed. The outer pools were filled with Ringer's fluid, a measured constant current was passed between them and hence axially through the canal, and the resultant voltage drop was recorded. The axial resistance of the canal was thus obtained and, when length and diameter were measured, R_i was determined.

Solutions

The Ringer's solution used in the present investigation was based on the analysis of *Raia* serum by Murray & Potts (1961) and had the following composition (mM): NaCl 287, KCl 4.0, $CaCl_2$ 1.0, $MgCl_2$ 1.0, $NaHCO_3$ 2.5 and urea 444 (26.6 g/l). pH was adjusted to 7.4 by the addition of up to 0.5 mM NaH_2PO_4.

The isotonic sucrose solution was 1.07 M (366 g/l) and had a resistivity of the order of 10 Ω cm as measured at 1 000 c/s.

Computations

Numerical solutions of eqn (18) were computed with an automatic digital computer (IBM 1401) at the Computations Division, Karolinska Institute.

RESULTS

Passive spread of potential

Figure 6 illustrates the spatial and temporal distribution of potential in an ampullary canal in response to a rectangular anodal voltage pulse,

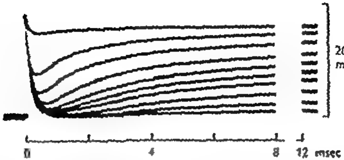

Fig 8 Experimentally recorded response after removal of the ampulla in the same canal as that of Fig 6 Individual traces superimposed in the experiment are from above downward the responses at distances 10 9 [illegible] 1 cm respectively from the cut end. Anodal step applied at 10 cm distance Time scale interrupted and response deleted between 8 and 12 msec The transient in the first msec is due to current through the electrode wall capacity 10.5 C

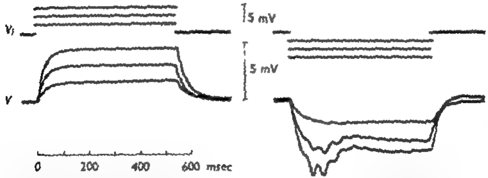

Fig 9 Response (V_2) recorded in an ampullary canal 0.5 cm from the ampulla to rectangular anodal (left) and cathodal (right) voltage pulses (V_1) applied at the other end Canal length 10 cm *R* [illegible] 10 C

lated end which was nearly short-circuited by the shunt across the stimulator output In the case of anodal pulses transients of a similar but lesser kind were sometimes seen after the break They were not observed in either case when the ampulla was removed The response to anodal pulses was preferable for the purpose of the present experiment but the cathodal response was useful as an indication of the condition of the preparation the absence of oscillations was generally accompanied by a marked spatial decline in passive response

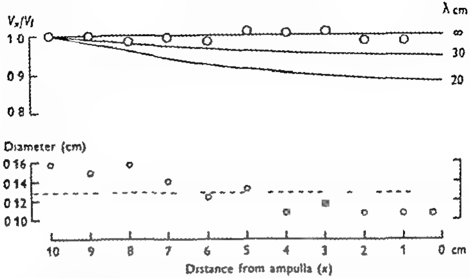

Fig 7 Steady state potential (upper graph) and canal diameter (lower graph) plotted against distance from the experiment of Fig 6 Upper ordinate potential expressed as a fraction of the applied potential Circles represent experimental points continuous curves are theoretical potential distributions from eqn (1 10) with λ infinite 30 cm and 20 cm respectively In the lower graph the horizontal dashed line represents the average canal diameter

in this canal was at least 30 cm and that the voltage gradient at $x = 0$ was practically zero The theoretical curves for $\lambda > 30$ cm lie too close together to permit a more exact estimate of λ with the available length of canal

The above observation was readily reproduced provided that the ray was in good condition (evidenced by a spirited resistance to being removed from the aquarium) that the ampullary canal was not anywhere punctured during dissection that the capillary electrode occupied not more than 10% of the cross section of the canal and that its tip in advancing did not scrape the wall of the canal in which event there was an immediate drop in the recorded response Inserting the capillary into or beyond the narrow neck of the ampulla generally had a similar deleterious effect which is why the response was not recorded at $x = 0$

When the ampulla was amputated thereby short circuiting the canal at $x = 0$ the steady state potential declined almost linearly with distance and the rise time of the transient was drastically reduced (Fig 8)

The above responses were obtained to anodal pulses When cathodal pulses of more than a few hundred μV were applied the records were disturbed by oscillatory transients They were greatest near the ampullary end of the canal (Fig 9) and decreased in amplitude towards the stimu

Table I Electrical constants in ten ampullary canals

Ray No	Canal No	Diameter cm	Temp C	r M Ω	R_m M Ω cm²	λ cm	c μF	C_m μF/cm²	τ_m sec
1	1	0 099	10	7 3	4 8	67	0 30	0 46	2 2
2	2	107	14	7 4	5 6	66	27	30	2 0
	3	108	14	5 6	3 8	58	29	41	1 6
3	4	103	11	8 4	5 4	67	31	48	2 6
4	5	115	10	3 4	3 1	61	36	40	1 2
5	6	094	11	16 8	9 9	87	24	44	4 0
	7	130	13	7 8	6 4	84	31	77	5 4
6	8	120	10	7 1	5 4	73	37	40	2 6
7	9	136	12	11 8	10 4	107	70	4	4 5
8	10	117	10	7 7	5 6	73	3	44	2 4
Average		0 116	1	8 4	6 0	74	0 31	0 43	5 4

Ray No 1 was *R batis* the others were *R radiata* Temperatures refer only to resistance measurements capacities were measured at 7—1 C Values of λ were calculated with $R = 30.5\ \Omega$ cm As r and c were measured in a 5 cm length of canal $r_m = 2r$ M Ω/cm and $c_m = (c/\ )\ \mu$F/cm

into pool C a marked increase in transmural current began after 30 sec and reached a plateau in 3 min when the measurements in Fig 10 B were made (filled symbols) The canal was then transected in pool C and the measurements repeated (open symbols) Both plots were fitted by the straight line representing a resistance of 3 8 kΩ which was evidently the value of r This experiment demonstrated that the measured resistance r was in the wall of the canal in pool C and that s was small enough to be ignored in the calculation of R_m Treatment with saponin was not routinely employed to measure r however as its value was obtainable from equ (2 3) without destroying the preparation

In the example of Fig 10 the canal diameter was 0 120 cm and R was therefore 5 4 M Ω cm The data from all canals in the series are given in Table I

Mural resistance was found to diminish with increase in temperature and the effect of warming the canal above 15°C was usually irreversible unless the canal was quickly re-cooled A reliable estimate of the temperature dependence of R_m was therefore obtained in but three canals (Nos 1 6 & 7) in which the Q_{10} was 1 5 2 4 and 2 4 respectively in the range 10—20 C

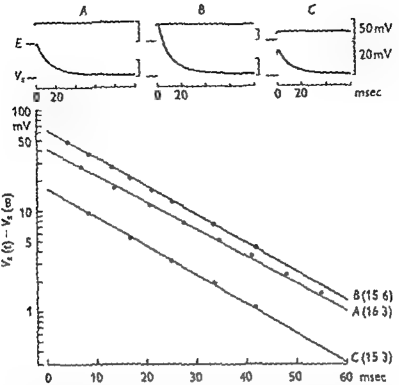

Fig. 11. Estimation of mural capacity from time constant τ_s. Above: three responses (V_s) to voltage steps (E). Note differences in time and voltage calibrations. Below: as ordinate, V_s minus steady state potential, in mV, on a logarithmic scale; as abscissa, time in msec. Straight lines fitted by eye. Values of τ_s for each graph are given in parentheses. Temperature and other data in Table II. Canal 3, *R. radiata*.

Measurement of mural capacitance C_m

When the transient portion of the response V_s was examined on an expanded time base, it was found that the rise time of the wavefront was less than 0.5 msec, indicating that the canal wall outside pool C was charged up rapidly enough that the use of the equivalent circuit of Fig. 5 and hence eqn. (22) involved no serious error. Furthermore, the response of the saponified canal indicated that the capacity measured by the present method was almost entirely attributable to the canal wall in pool C.

Figure 11 and Table II illustrate the calculations used in obtaining C_m in one canal. The figure reproduces 3 experimental records of $V_s(t)$ and the corresponding semilogarithmic plots of $V_s(t) - V_s(\infty)$. Fitting straight lines to the plots gave the values of the time constant τ_s and by extrapolation the values of $V_s(0)$, as the latter were not easily read from

Table II Estimation of mural capacity

Record No	Temp °C	r_e kΩ	r MΩ	r kΩ	τ_p msec	c μF	C_m μF/cm
11 A	14	(15.2)	(5.6)	11.5	16.3	0.29	0.43
11 B	14	(15.2)	(5.6)	12.3	15.6	0.27	0.40
11 C	17	(15.[illegible])	([illegible].2)	10.2	15.3	0.28	0.43
					Average values	0.28	0.41

Ray No canal No 3 Dimensions of canal 20 × 0.109 cm surface area 6.84 cm² Data in parentheses from other records

the original records Values of r_e were then calculated with eqn (2.3) Measurements of the corresponding values of r_e and r were made from steady state records All the data are given in Table II As r was very large compared to $(r_e + r)$ eqn (2.2) reduces to

$$\tau \simeq c(r_e + r)$$

from which the value of c was obtained This reduced form of the equation was applicable in the other experiments as well the results are collected in Table I

In the example of Fig. 11 records 11 A and 11 B were taken early in the experiment record 11 C about 2 hours later and at a higher temperature These and the other data in Table I indicate that the mural capacity was to a considerable extent independent of the level of the mural resistance and of temperature in the range 7—17°C

Measurement of core resistivity R_i

Core resistivity was measured in three canals and the data obtained are given in Table III The average value of R was 30.5 Ω cm at 10°C or as

Table III Core resistivity in three ampullary canals from R radiata

Ray No	Temperature °C	Canal length cm	Diameter (average) cm	r Ω/cm	R_i Ω/cm
9	9.5	10.5	0.130	2150	28.5
10	10.0	4.9	0.165	1480	31.6
11	9.5	7.2	0.145	1660	31.3
Average	9.7				30.5

conductivity 0·033 mho/cm. If the Q_{10} of the conductance of the jelly is the same as that of sea water (1·26, 10—20°C, Krummel, 1907), their ionic compositions being very nearly identical (Murray & Potts, 1961), then at 20°C the core conductivity is 0·042 mho/cm, which agrees with the value of 0·040 mho/cm obtained directly by the latter workers.

Comparison of theoretical and experimentally recorded responses

With the measured values of the electrical constants in hand, it was possible to compute appropriate numerical solutions of eqn. (1·8) or (1·10) and to compare them to experimentally recorded responses. Figure 12 presents such a comparison. In view of the fact that the response and the constants were obtained from separate experiments, the overall coincidence of the two sets of curves is reasonably good and may be favourably compared to the fit obtained by Hodgkin & Rushton (1946) when they applied the equations for an infinite cable to the passive response of an axon.

The divergence of the two families of curves is largely attributable to the fact that the real canal is not a perfect cylinder. The theory assumes canal diameter and hence λ to be constant, but the real canal tapers irregularly from about 0·16 cm at the distal end to about 0·11 cm at the neck of the ampulla (Fig. 7). The effect is a progressive reduction in the value of λ for given values of R_m and R_i. As may be seen in Fig. 12, an improved fit is obtained at $x = 1$ cm when the theoretical value of λ is reduced to 72 cm, which is approximately equivalent to the observed reduction in diameter. A more rigorous but complicated test would involve treating the canal parameters as functions of length, as in the analogous case of Heaviside's Bessel cable (Carslaw & Jaeger, 1948, p. 209; cf. Sten-Knudsen, 1960).

Although the more general solution (1·8) was employed in this example, an almost equally good fit is obtainable with eqn. (1·11), for the two are very nearly coincident when λ is more than five times canal length.

DISCUSSION

In the Introduction two possibilities were entertained that would allow adequate electrical transmission along an ampullary canal. The one, that the canal is a passive and well insulated conductor, is strikingly borne out by the present observations: the ampullary canal behaves like an ideal submarine cable that is terminated by an open circuit at the receiving end. The other possibility, that the canal *per se* is excitable, may be excluded by the finding that the mural resistance obeys Ohm's law for both anodal

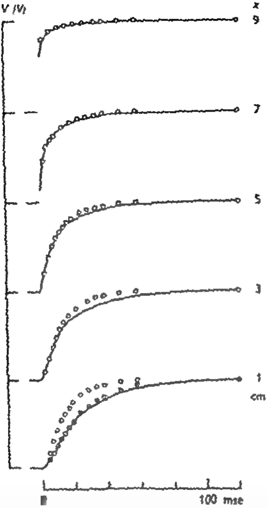

Fig 17 Comparison of theoretical and experimentally recorded responses of an ampullary canal to a voltage step Abscissa time in msec Ordinate potential expressed as a fraction of the voltage at stimulated end ($x = 10$ cm) Ampulla at $x = 0$ Continuous curves traced from the records shown in Fig ▮ Theoretical points (○ ○ ○) computed with eqn (1 9) for $\lambda = 90$ cm and $\tau_p = ?4$ sec Additional curve (○ ○ ○) at $x = 1$ cm computed for $\lambda = ??$ cm $\tau_p = ?4$ sec

and cathodal currents The oscillatory transients observed in the intact ampullary canal in response to cathodal stimuli must arise in the ampulla they represent electrical activity either in the receptor cells the afferent nerve terminals or both Apparently similar oscillations were recorded by Murray (1965) on thrusting an electrode into the pore end of an ampullary canal in an undissected just-dead ray

When compared with the electrical resistance of other epithelia, that of the canal wall, which averaged [illegible] MΩ cm^2, is uniquely high. It is very much higher than the resistance of elasmobranch skin. Bennett (1961) gives a mean of about 1 000 Ω cm^2 for *Torpedo occidentalis* and one value of 370 Ω cm^2 for *Squalus acanthias*; in 3 specimens of *R. radiata* the d.c. skin resistance was 400—2 800 Ω cm^2, average 900 Ω cm^2 (Waltman, unpublished). The canal mural resistance is two or three orders of magnitude higher than any epithelial resistance previously reported. Even the myelin sheath is leaky by comparison (0.1 MΩ cm^2, Tasaki 1955), its virtue as an insulator for nerve being of course its very low capacitance (0.005 μF/cm^2) owing to the concentric arrangement of over 200 membranes, each of about 1 μF/cm^2, in electrical series. Yet the mural capacitance of the ampullary canal, about $1/2$ μF/cm^2, would be accounted for by only two such membranes, as may be provided by a single layer of cells. This question and other morphological considerations are taken up in Part II.

Although there is as yet no behavioural evidence that the ampullary canals confer an electric sense upon fish that possess them, the electrical sensitivity of the ampullae and the cable properties of the canals support the surmise that this is their function. Until the natural stimuli to which they normally respond have been identified, the present analysis provides criteria for judging the effectiveness at the ampulla of any electrical input. The theoretical analysis and experimental recording of the canal response were made with the condition of negligible impedance at the input; the large surface and relatively low resistance of the skin and gills would ensure that a similar boundary condition obtains at the input of the canal *in vivo*. It follows that a steady potential applied at the pore is dropped almost entirely across the ampulla and that, for a uniform d.c. gradient outside the fish, the longer the canal, the greater is the effective stimulus.

When the voltage across the input is not stationary but changes in time, the canal will respond like a low pass filter. As its transmission characteristics are functions of length, in this case the longer the canal, the more attenuated and retarded is the effective stimulus. It may be shown by graphical subtraction (cf. Thomson 1856) that when a 10 msec rectangular pulse is applied to the distal end of a 10 cm hyoid canal, the peak of the wave received at the ampulla will be down 10 db (to 30% of the input) and lag 16 msec after the time of onset. The discharge of the caudal electric organ of the ray is roughly trapezoidal in shape but has a plateau of at least 300 msec duration (Albe-Fessard 1950) and will escape serious loss. The spike potentials detected by Kleerekoper & Sibakin (1956) near the head of a sea lamprey would clearly be distorted except in shorter

canals. An estimate of the comparative filtering effects of canals of different lengths is given by the following turnover frequencies (3 db down) evaluated from the theoretical steady state response at the ampulla to an alternating voltage input: for a 1 cm mandibular canal (0·04 cm diameter) about 300 c/s; for a 10 cm hyoid canal (0·12 cm), 9 c/s; for a 20 cm hyoid canal (0·12 cm), 3 c/s.

As the canals cover most of the surface of the head, diverging in all directions from the ten encapsulated groups of ampullae, the arrangement could provide information about the strength and configuration of an electric field around the fish. The dependence of the transient response upon canal length may suggest that the pipe-organ array of canals performs a kind of frequency analysis as well, but it is more likely that the operation of the receiving system does not depend upon time discrimination by individual ampullae. The latter is supported by Murray's (1962, 1965) observations that in respect of the afferent spike discharge to pulse train stimuli the mandibular ampullae respond as if the stimulus is d.c. of equivalent charge per sec. A similar conclusion was reached on the basis of behavioural responses for the electric receptors in the teleost *Gymnarchus* (Machin & Lissmann, 1960).

Gymnarchus employs its electroreceptors to map the distribution of an electric field set up by the emissions of its electric organ; objects entering this field are detected by the distortions they cause in it (Lissmann & Machin, 1958). Although *Raia* would appear to possess sensory equipment adequate for a similar analysis of the environment, its caudal electric organ, at least in captive specimens, is silent unless the skin is irritated (Fessard, 1958). And other elasmobranchs and the teleost *Plotosus* (Friedrich-Freksa, 1930) have ampullae but not electric organs. Furthermore, behavioural experiments like those employed to demonstrate the electric sense in *Gymnarchus* have not apparently been successful with the skate (Lissmann, 1963). The demonstration in a species of dogfish (Dijkgraaf & Kalmijn, 1963) that motor responses to a potential gradient of 3 μV/cm near the head are abolished by ampullary nerve section confirms the electrical sensitivity of the ampullae, but the nature of the evoked reaction ('*Augensunkern*') and the condition under which it occurs ('*Zustand des Eindösens*') give no clue to the significance of this sensitivity in nature. There is in fact only the suggestive observation of Steven (1932) based on a comparison between day and night hauls by long line fishing that *Raia*, unlike turbot, are not sight feeders but rely on some other sense in foraging. The existence of an electric sense in elasmobranch fish must

When compared with the electrical resistance of other epithelia, that of the canal wall, which averaged 6 MΩ cm², is uniquely high. It is very much higher than the resistance of elasmobranch skin. Bennett (1961) gives a mean of about 1 000 Ω cm² for *Torpedo occidentalis* and one value of 370 Ω cm² for *Squalus acanthias*; in 3 specimens of *R. radiata* the d.c. skin resistance was 400—2 800 Ω cm², average 900 Ω cm² (Waltman, unpublished). The canal mural resistance is two or three orders of magnitude higher than any epithelial resistance previously reported. Even the myelin sheath is leaky by comparison (0·1 MΩ cm², Tasaki 1955), its virtue as an insulator for nerve being of course its very low capacitance (0·005 μF/cm²) owing to the concentric arrangement of over 200 membranes each of about 1 μF/cm² in electrical series. Yet the mural capacitance of the ampullary canal, about ½ μF/cm², would be accounted for by only two such membranes as may be provided by a single layer of cells. This question and other morphological considerations are taken up in Part II.

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II The Fine Structure of the Ampullary Canals of Lorenzini in *Raia*

INTRODUCTION

The electrical properties of the ampullary canals of Lorenzini described in the first part of this report were considered sufficiently exceptional to occasion an attempt to identify them with the histological structure of the organ. The literature reveals that although Lorenzini (1678) considered the wall of the canal thicker than what was appropriate for a simple duct, it is in fact quite thin in the light microscope. Müller (1851), Leydig (1852), Boll (1868), Todaro (1870), Merkel (1880), Fritsch (1888), Peabody (1897), Retzius (1898), Forssell (1899), Metcalf (1915, 1916), Dotterweich (1932) and Kantner, König, & Reinbach (1962) all agreed that the canal wall in various elasmobranch species was a single layer of squamous epithelium in a fibrous sheath. About the epithelium lining the ampulla there was less accord but many of the authors were able to distinguish in the lining of the ampullary alveoli cells of two kinds alternating in a single layer: piriform flask cells and inverted pyramidal cells, the apices of the former debouching on the lumen between the bases of the latter. Which of these two cell types mediated the suspected sensory function was a point of dispute, but Barets & Szabo (1962), in an electron microscopical study addressed to this question, clearly showed that only the flask cells are innervated, thereby confirming the descriptions given by Retzius and Metcalf. Finally, the so-called centrum cap or *Zentralplatte*, which is the central anvil-shaped elevation in the very bottom of the ampulla, was said to be covered by a simple cuboidal or columnar epithelium that was not innervated.

The description obtained from these accounts suggests that the wall insulation is the property of a simple epithelium but does not rule out the existence of some other dielectric barrier. This warranted a microscopical examination of the canal wall.

The electrical measurements indicated that the ampulla itself was also well insulated. As the ampullary receptors are excited by electrical stimuli

they should lie across the insulating layer. Yet in contrast to the situation described earlier, Barets & Szabo (1962, Pl. I a) show the innervated flask cells separated from the lumen by the pyramidal cells. If the resistance of the pyramidal cells is high, the arrangement would appear to be inconsistent with the function of the flask cells as electroreceptors. A re-examination of the fine structure of the ampulla was therefore justified.

MATERIAL AND METHODS

Ampullary canals of Lorenzini from the hyoid group in *Raia radiata*, *R. clavata* and *R. batis* were examined. The fish were kept in running sea water at 12°C or less; dissection after pithing was conducted in a cold room at 11°C.

Light microscopy

Ampullae were exposed by opening the hyoid capsule and stained *supravitam* with methylene blue by Unna's method (Romeis, 1928).

Mounts of the canal epithelium were made by opening a short length of canal, pinning it out on wax and silvering by Ranvier's method (Romeis, 1928) with the exception that the silver nitrate (0.5%) was reduced with ordinary photographic developer ('Promicrol', May & Baker) and fixed in hypo.

A few specimens were fixed in Bouin's fluid and wax sections stained by Bodian's protargol method.

Electron microscopy

Single ampullae and cm lengths of canal were fixed by immersion in one of three fixatives:

1) osmium tetroxide 1—2% for 1—2 hr
2) potassium permanganate 0.5—1.5% for 15—60 min
3) glutaraldehyde 2.5 or 4% for 2—10 hr followed, after washing, by 1% osmium tetroxide for 1 or 2 hr.

The fixatives were dissolved in elasmobranch Ringer's solution buffered to pH 7.2—7.6 either with bicarbonate as in the recipe on p. 16, or with 0.1 M phosphate (in which case Ca^{++} was omitted from the Ringer's) or 0.1 M cacodylate. Fixation and washing in buffered Ringer's or distilled

water were done at 2—10°C. Dehydration was carried out in a graded ethanol series and embedding in Epon.

Thin sections were cut on glass knives in an ultramicrotome (Ultrotome, LKB Produkter AB) and picked up on Formvar coated grids. The permanganate fixed and some osmium fixed sections were examined unstained; the others were treated with lead citrate (Reynolds, 1963; Venable & Coggeshall, 1965) alone or preceded by uranyl acetate in water. Examination was made in an Elmiskop I (Siemens) operated at 60 kV and plates were exposed with objective apertures of 20 or 30 μ at magnifications of 1 000 to 40 000 times as read from the ammeter provided in the microscope. No correction was made for variations in magnification described by Elbers & Pieters (1964) or for tissue compression, although all sections of the canal were cut in the same direction (against the lumenal surface through the axis of the canal). The figures were made by photographic enlargement and calibrated by comparing the print and negative in two normal axes.

OBSERVATIONS

Light microscopy

The gross anatomical and histological features of the ampullary canals are well seen with the light microscope in methylene blue stained preparations (Figs. 13, 14 and 15). The cylindrical canal (which at the skin surface is open to the sea) terminates proximally in a dilated alveolate bulb, the ampulla, over whose surface ramify the unmyelinated terminal branches of medullated axons from the hyomandibular branch of the facial nerve. Blood capillaries are rare along the canal but comparatively numerous about the ampulla. When the stain is permitted to act long enough, cells in the canal wall become apparent (Figs. 13 and 14). As mentioned above, previous workers recognized but a single layer of cells and indeed, in histological sections of wax embedded material the canal epithelium appears only one cell thick. However, when the canal is opened flat and treated with silver nitrate, two layers of cells are discerned; the cellular outlines in one are thick and tortuous, in the other thin and straight (Fig. 16). Under an oil immersion objective the two patterns may be focussed separately (Figs. 17 and 18); the thick borders are more superficial, i.e. lumenal, the thin borders are deeper. The difference between the planes of focus was 0.5 μ, as measured with a micrometer dial bench gauge. In several mounts only one set of cell borders was seen but when the

they should lie across the insulating layer. Yet, in contrast to the situation described earlier, Barets & Szabo (1962, Pl. I a) show the innervated flask cells separated from the lumen by the pyramidal cells. If the resistance of the pyramidal cells is high, the arrangement would appear to be inconsistent with the function of the flask cells as electroreceptors. A re-examination of the fine structure of the ampulla was therefore justified.

MATERIAL AND METHODS

Ampullary canals of Lorenzini from the hyoid group in *Raia radiata*, *R. clavata* and *R. batis* were examined. The fish were kept in running sea water at 12°C or less; dissection after pithing was conducted in a cold room at 11°C.

Light microscopy

Ampullae were exposed by opening the hyoid capsule and stained *supra vitam* with methylene blue by Unna's method (Romeis, 1928).

Mounts of the canal epithelium were made by opening a short length of canal, pinning it out on wax and silvering by Ranvier's method (Romeis, 1928) with the exception that the silver nitrate (0.5%) was reduced with ordinary photographic developer ('Promicrol', May & Baker) and fixed in hypo.

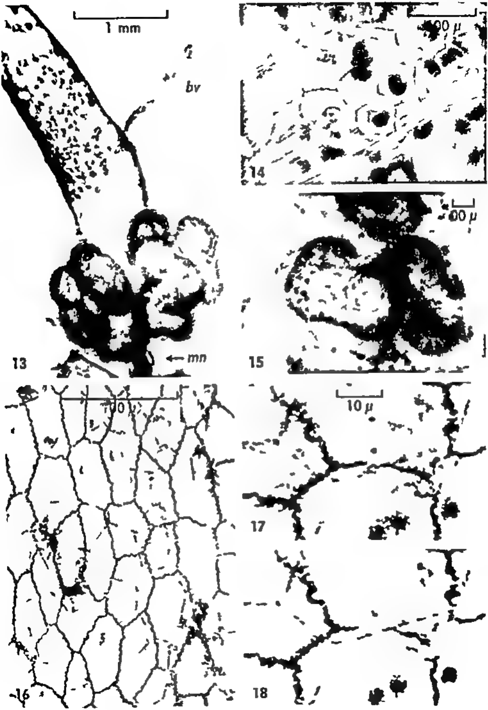
A few specimens were fixed in Bouin's fluid and wax sections stained by Bodian's protargol method.

Electron microscopy

Single ampullae and cm lengths of canal were fixed by immersion in one of three fixatives:

1) osmium tetroxide, 1—2%, for 1—2 hr;
2) potassium permanganate, 0.5—1.5%, for 15—60 min;
3) glutaraldehyde, 2.5 or 4%, for 2—10 hr, followed after washing by 1% osmium tetroxide for 1 or 2 hr.

The fixatives were dissolved in elasmobranch Ringer's solution buffered to pH 7.2—7.6, either with bicarbonate as in the recipe on p. 16, or with 0.1 M phosphate (in which case Ca^{++} was omitted from the Ringer's), or 0.1 M cacodylate. Fixation and washing in buffered Ringer's or distilled


1 mm
bv
mn
13
100 μ
14
100 μ
15
100 μ
16
10 μ
17
18

cm² It follows that if a continuous intercellular gap of 20 Å or more can be resolved in the electron microscope from lumen to epithelial base then it is unlikely that the epithelium is an effective insulator

Electron microscopy

General organization The wall of the ampullary canal may be divided into three principal regions (seen in a single section in Fig 19) the canal the ampullary alveolus and between them a narrow marginal zone

The epithelium lining the canal is 1—2 μ thick and made up of two distinct layers of cells It rests on a lamina of collagenous connective tissue

The alveolar epithelium is about 15 μ thick and contains the two kinds of cells previously described the innervated flask cells called here receptor cells and the un innervated pyramidal cells called accessory cells

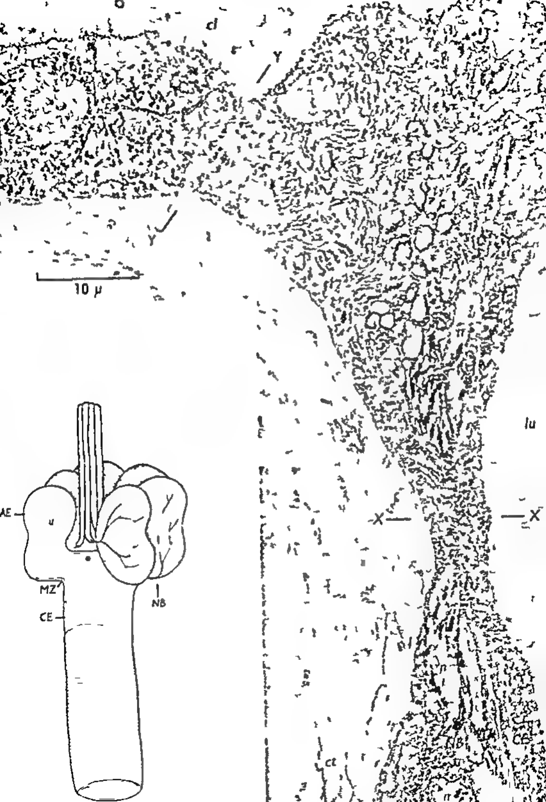
The marginal zone forms the rim of each alveolus In Fig 19 it is seen to receive from below a bundle of unmyelinated axons wrapped in sheath cells the basement membrane of which is continuous with that of the canal and ampulla The axons apparently pass through the marginal zone to innervate the alveolar receptor cells although direct continuity was not seen, nerves were never found entering the alveolar epithelium directly

The centrum cap may be considered as a fourth region but in the one ampulla in which it was examined its structure appeared the same as that of the canal except that the epithelial cells were deeper (Fig 28)

The lumen of the canal and ampulla which in life contains a stiff homogeneous transparent jelly in the electron microscope appears filled with scattered strands of electron-dense material that are best seen in glutaraldehyde fixed tissue In the canal these strands radiate for short distances from condensed patches against the free surface of the epithelium the

Fig 19 Section through the rim of an ampullary alveolus as indicated in the inset drawing showing the transition from the canal epithelium (*CE*) through the marginal zone (*MZ*) to the alveolar epithelium (*AE*) the divisions being marked approximately at A—— A and B —— B respectively A bundle of naked axons (*NB*) wrapped in a sheath cell (*sc*) joins the canal at a low angle apparently having circled the margin of the alveolus (as shown in the inset) and penetrates the marginal zone The lumen (*lu*) appears homogeneous except above the alveolar epithelium where extracellular particles in the corpuscular layer (*cl*) are evident Loose connective tissue (*ct*) supports the wall *n* Nerve *P clavata* (Osmium-fixed stained with lead citrate × 2,500)

Inset Diagrammatic representation of an ampulla of Lorenzini (cf Fig 13) partially cut away to show the site of the *section (dashed rectangle) seen in the micrograph. The nerve* fibres lose their myelin under the centrum cap ( ) pass between the alveoli and then ramify over them (cf Metcalf 1916 Pl 2) Letter references as above



b
d
Y
Y
10 μ
lu
X
X
CC
CB
AE
u
MZ
CE
NB

subjacent cytoplasm then looks denser than elsewhere (Fig 29) In the lumen of the alveolus and nowhere else an aggregate of extracellular bodies overlies the epithelium to a depth of several μ and is called the corpuscular layer

Relevant features of the canal and ampulla will now be described in more detail

The wall of the canal Figure 20, a longitudinal section through the full thickness of the canal wall shows the two layers of cells in the epithelium They are separated by a basement membrane from a deep lamina of connective tissue containing regularly oriented collagen fibres The fibroblasts do not form an integral layer

The superficial and the deep cells of the epithelium differ in the nature of their lateral connexions The side of each superficial cell is comparatively straight and at the lumenal end of its apposition with the side of an adjacent cell, it makes a 'tight junction the outer leaflets of the contiguous plasma membranes appear to fuse and no intercellular space is seen (Figs 21 22 24 and 26) The width of the resulting five layered configuration (3 dark separated by 2 light layers) is measurably less than the sum of the thicknesses of the two trilaminar membranes beyond the junction A continuous tight junction of this kind generally extends for 0 2 to 0 5 μ below the lumenal surface Beyond this portion of the junction the opposed membranes may fuse intermittently down to the base of the cell layer Although seen after the other fixatives this was best demonstrated in permanganate-fixed tissue (Figs 24 25 and 26) One or two desmosomes may be encountered in the same region

Unlike the superficial cells adjacent deep cells interdigitate widely and their lateral surfaces are always separated by an intercellular gap of 100 Å or more Desmosomes are rarely seen between them In favourable sections the path between two adjacent cells may be followed without interruption through the cell layer in the example of Fig 23 the path length is about 4 μ A space of 100 Å or more separates the adjoining surfaces of superficial and deep cells and desmosomes join them at regular intervals

Other differences may be noted between deep and superficial cells Large oval or irregular spaces are often seen in the cytoplasm of the superficial cells both in the canal proper and in the centrum cap A trilaminar membrane like the plasma membrane defines the perimeter of each space although it is not always completely evident The spaces are usually disposed towards and close to the lumenal surface In osmium fixed tissue they have the same density as the lumen (Figs 21 and 23) in glutaraldehyde fixed tissue

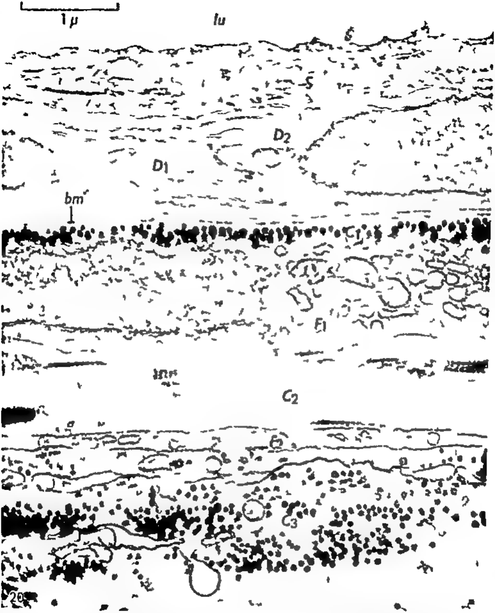


Fig 20 Longitudinal section through the full thickness of the canal wall showing the lumen (*lu*) a cell of the superficial layer (*S*) two cells of the deep layer (*D* *D*) with a portion of nucleus in each and a meandering path between The basement membrane (*bm*) separates the epithelium from the connective tissue below two laminae of encircling collagen fibres (C C_3) one of longitudinal fibres (C) and portions of at least two fibroblasts (F F_2) Note the predominance of rough surfaced cisternae in the cytoplasm of superficial cell *S* compared to deep cells *D* and *D* *R radiata* (Osmium fixed stained with uranyl acetate and lead citrate × 24 000)

they contain a web of strands like those in the lumen (Fig 28) In only a few sections were surface invaginations seen that could be interpreted as the communication of such a space with the lumen Rough surfaced cisternae of the endoplasmic reticulum are also especially common in the superficial cells (Fig 20) and the Golgi complex is prominent (Fig 27)

In an experiment described in Part I (p 20), it was shown that treatment with 1% saponin in Ringer's solution irreversibly reduced the mural resistance from over 5 M Ω cm to practically zero Sections from the treated canal (Fig 31) and from another (Fig 30), kept in Ringer's solution at 6°C during the experiment (about 4 hours) indicate that in the treated canal the cell membranes are either dissolved or badly disrupted No distinct change in the basement membrane is seen

The marginal zone Tight junctions and desmosomes characterize the lumenal ends of the lateral cell contacts in the marginal zone Below this region a gap of about 100 Å is seen and the course of the intercellular pathway is long and tortuous giving a labyrinthine pattern to sections through the zone (Fig 19)

The free surface of the epithelium is roughened by many microvilli The cytoplasm of the epithelial cells and of the axons they surround are especially rich in mitochondria

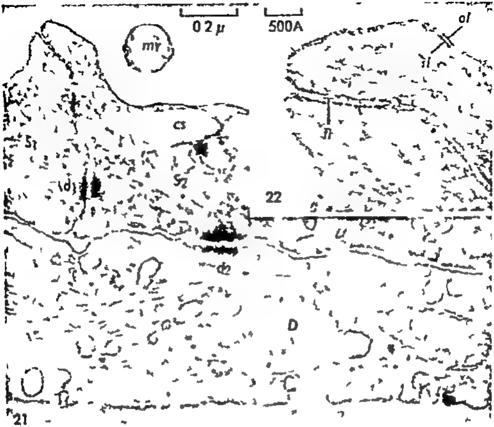
The ampullary alveolus The disposition of the receptor and accessory cells in the alveolar epithelium is shown in Fig 32

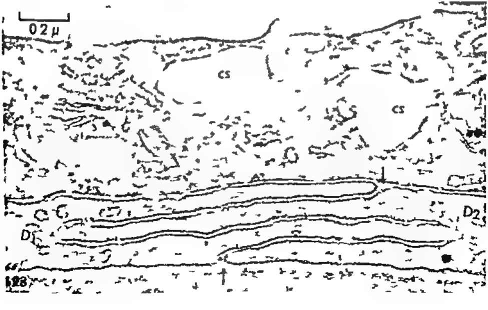
The apex of each receptor cell reaches the lumen of the alveolus and it bears a cilium The cilium is about 0 3 μ in diameter and probably not less than 5 μ long The axoneme seen in cross sections contained 9 double fila

Fig 21 Section through canal epithelium showing the side contact between two superficial cells (S_1, S_2) including the juxtalumenal tight junction (*t*) and a desmosome (d_1) The path between the superficial cells and the deep cell (*D*) appears open except at another desmosome (d_2) *cs* Cytoplasmic space bound in part by a unit membrane *mv* microvillus seen in cross-section or pinched off *R radiata* (Osmium fixed stained with uranyl acetate and lead citrate ×65 000)

Fig 22 Enlargement of the tight junction in Fig 21 showing the fusion lamina (*fl*) inner lamina (*il*) and outer lamina (*ol*) of the plasma membrane (× 100 000)

Fig 23 The lateral relations of two cells in the deep layer of the canal epithelium (*D* *D*) Extracellular space is continuously open between the arrows A pair of cytoplasmic spaces (*cs*) are seen in the overlying superficial cell (S) The section is from the same ribbon as that seen in Fig 21 *R radiata* (Osmium fixed stained with uranyl acetate and lead citrate × 60 000)


0 2 μ
500A
mr
ol
cs
St
St
fl
d
22
u
D
21


0 2 μ
cs
cs
D2
D1
123

ments without arms the pattern was 8 + 1 in 6 cilia In one case 3 cross sections apparently of the same cilium were found in a single section (Fig 38) two of the patterns are 8 + 1 but the third may be 9 + 0, which would correspond to a similar transition in pattern demonstrated by Dahl (1963) in serial sections of cilia from rat hippocampus Neither rootlet fibres nor an accessory centriole were identified The ciliary membrane was wrinkled or fragmented and was sometimes seen detached and empty of cytoplasm

Synapses occur on the bases of the receptor cells (Figs 34 and 39) Their structure is identical to that described by Barets & Szabo (1962) for the ampullae in *Torpedo* their Fig 1 a diagrammatic representation of the synapse in three dimensions equally well depicts the arrangement in *Raia* Each synapse is formed by the projection of a long narrow ridge of receptor cell into a corresponding gutter in the afferent axon—the arrangement resembles the tongue and groove joint between match boards On either side intrusive projections of accessory cells delimit the apposition of receptor cell and axon to the locus of the tongue and groove The width of the synaptic interspace in the present material was 100—200 Å A ribbon about 250 Å thick and 1—2 μ wide inserts edgewise into the tongue of the receptor cell throughout its length which may exceed 4 μ Both surfaces of the ribbon are covered with a single layer of vesicles that are round or slightly elliptical in outline Their outside diameters range from 300 to 550 Å A similar population of vesicles is found free in the cytoplasm

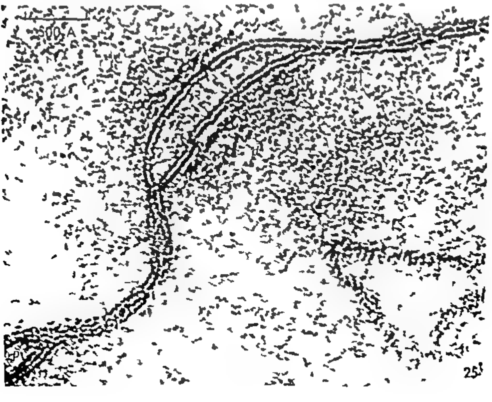
Except where they debouch on the lumen the receptor cells are enclosed and isolated from each other from intraepithelial axons (except at the synaptic loci) and from the basement membrane by a surround of accessory cells Microvilli project from the surface of each accessory cell and rough and smooth surfaced cisternae are seen in the cytoplasm

The lateral contacts between three accessory cells (Figs 35 and 36) and between an accessory cell and a receptor cell (Fig 37) include tight junctions at their lumenal ends elsewhere down to the base of the epithelium in the former case no interdigitation takes place and the gap is 100 Å or more Desmosomes were not seen in the alveolar epithelium

Fig 24 Section through the lateral junction between two superficial cells in the canal epithelium (S S) Fusion of the contiguous plasma membranes occurs near the lumen (L) and intermittently through the region of a desmosome down to the surface of a deep cell (D) Another superficial cell intrudes at p *R radiata* (Permanganate fixed × 100 000)

Fig 25 Enlargement of part of Fig 24 showing the desmosome (d) part of an intruding cell (p) and intermittent formation of the five layered junctional complex (arrows) (× 300 000)


0.2 μ
lu
P
D
24


500 A
25

The corpuscular layer The layer of extracellular bodies that overlies the alveolar epithelium extends outwards from the lumenal surface for a distance of about 1 to 10 μ and ends abruptly The lumen beyond has its usual appearance Sections through the layer contain profiles of two kinds round or oval ones that are limited by a membrane and straight or slightly curved ones that look like bits of membrane on edge The rounded profiles are 200—1 200 A across the density of their contents varies but is greater than that of the lumen The bits are 400—1,200 A long The membrane limiting the round profiles is trilaminar and its thickness like the width of the bits is not appreciably different from that of the lumenal cell membrane in the same section In osmium fixed material, the layer may contain clumps of fine granules (Fig 33)

DISCUSSION

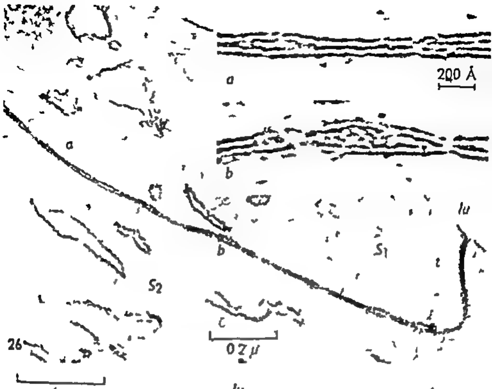
Identification of the wall insulation

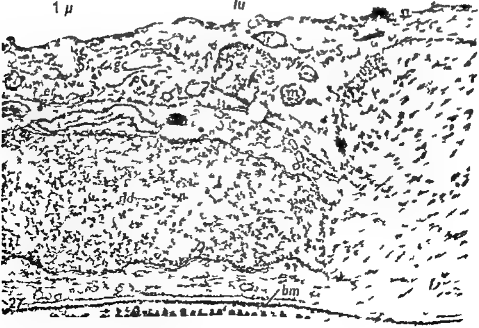
The electron micrographs of the canal epithelium show a space of at least 100 A between the deep cells of the epithelium but not at the lumenal ends of the junctions at least, between the superficial cells It is unlikely that cells so close together would react differently to the preparative procedures and it is not unreasonable therefore that the space observed between the deep cells exists *in vivo* Haemoglobin has been seen to enter similar intercellular spaces between kidney tubule cells (Farquhar & Palade 1963) The argument on p 32 would then exclude the deep cell layer as an effective insulator in the canal wall As the lamina of connective tissue is not structurally continuous two possibilities remain the superficial cell layer or the basement membrane insulates the canal

The basement membrane of the canal is continuous with that of the ampulla If the basement membrane had a high resistance, a transmural potential would be dropped across it and not across the receptor cells But the receptor cells are excited by transmural potentials and therefore the basement membrane should have a low resistance

Fig 26 Section through the lateral junction between two superficial cells in the canal epithelium (S_1 S_2) *lu* Lumen *t* tight junction The regions *a* and *b* are enlarged in the insets *R radiata* Permanganate fixed × 80 000 insets × 470 000)

Fig 27 Section through the canal epithelium showing a superficial cell (*S*) with its nucleus (*ns*) and a deep cell (*D*) with its nucleus (*nd*) *bm* Basement membrane *er* endoplasmic reticulum *gc* Golgi complex *lu* lumen *m* mitochondrion *x* unidentified *R radiata* (Osmium fixed stained with uranyl acetate and lead citrate × 2 000)


a
200 Å
b
a
b
lu
S_1
S_2
c
0.2 µ
26
1 µ


lu
bm
27

It appears then that the wall insulation is due to the superficial layer of cells. This would provide two cell membranes in series. Other cell membranes of similar thickness (~ 70 Å) have a capacity of about 1 μF/cm² (e.g. Thompson, 1964); two of them in series would give $^1/_2$ μF/cm². The wall capacity was found in Part I to average 0.4 μF/cm². The highest ohmic resistance reported for a single cell membrane in a physiological medium is 20 k Ω cm² in *Nitella* (Williams, Johnston & Dainty, 1964) but a 60 Å membrane prepared from phospholipid (Thompson, 1964) has about the same resistance as observed for the canal wall and a capacity of 1 μF/cm², indicating that the canal insulation is not incompatible with the properties of known biological dielectrics.

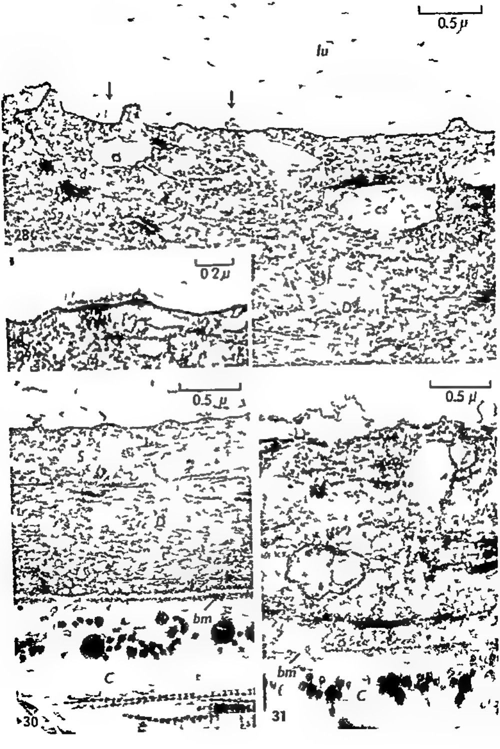
If the superficial cells insulate the canal, there must be no appreciable electrical leakage between them. An intercellular space wide enough by the argument on p. 32 to provide a conducting pathway was resolved between cells except in five layered tight junctions. *This leads to the conclusion that tight junctions in this epithelium are impermeable to ions.*

The insulation of the ampulla is now considered. The electrical constants were not directly measured in the ampulla proper, but because the intact organ behaved as if the ampullary termination was open circuited to *anodal potential steps, the ampulla must also be effectively insulated.* It was pointed out above that the sensory epithelium will be stimulated by a transmural potential only if the excitable structures are in parallel and not in series with the high resistance of the wall. The necks of the receptor *cells are collared by tight junctions* with adjacent accessory cells and the latter are themselves occlusively joined to each other by tight junctions at the same level. Receptor and accessory cells are therefore structurally in parallel and appear to satisfy the necessary condition.

Fig. 28. Section through the epithelium of the centrum cap. Scattered electron-dense strands fill the lumen and appear to radiate towards dense patches (arrows) against the lumenal surface of a superficial cell (S.). The extoplasmic space (ex) contains the same material in somewhat greater concentration. The cytoplasm of S. of another superficial cell (S.) and of a deep cell (D.) contain many fibres. *d*. Desmosome. *t*. tight junction. *R. radiata*. (Glutaraldehyde fixed, stained with lead citrate. $\times$ 20 000.)

Fig. 29. A dense patch against the free surface of a superficial cell in the canal epithelium and the strands radiating from it. *R. radiata*. (Glutaraldehyde fixed, stained with lead citrate. $\times$ 45 000.)

Figs. 30 and 31. Sections of untreated and saponin treated canal wall respectively. The experiment is described in the text. Note the disruption of the cell membranes and the persistence of the basement membrane (*bm*) in Fig. 31. *c*. Collagen. *D*. deep cell. *S*. superficial cell. *R. radiata*. (Glutaraldehyde fixed, stained with lead citrate. $\times$ 20 000.)


0.5 μ
lu
a
cs
28
0.2 μ
D
0.5 μ
0.5 μ
D
bm
C
30
bm
C
31

An experimental result may be given as evidence that the electrically excitable cells in the ampulla lie across the mural insulation. An ampullary canal was placed in Ringer's fluid and a sucrose gap set up across the cut end of the canal. The resistance looking into the canal in parallel with the gap resistance was 0.3 MΩ to an anodal (lumen positive) current of 6×10^{-8}A that was suddenly applied through a series resistance of 5 MΩ. A cathodal current of the same strength evoked a train of damped negative going oscillations and the steady state resistance fell to 0.06 MΩ. Spike discharges coincident with the wave fronts could be led from a filament of the ampullary nerve. As the mural resistance in the canal is ohmic the rectification occurred in the ampulla and the elements responsible, likely the receptor cell, must lie across the insulation in its wall.

The tight junction

Five layered tight junctions identical in structure to those found here have been described between cells in a great many other tissues; the extensive paper of Farquhar & Palade (1963) may be consulted for their demonstration in vertebrate epithelia and for a review of the literature. In Farquhar & Palade's terminology they form the juxtaluminal *zonula occludens*; this probably corresponds to zone II in Zetterqvist's (1956) description of the intestinal mucosa of the mouse, where Sjostrand (1963) has since resolved the five layered complex. Because tight junctions of this kind are found in sheets of cells separating media of different composition it has been suggested that they prevent the intercellular diffusion of ions and molecules (see Muir & Peters, 1962). Direct evidence of this was obtained by Muir & Peters and by Choi (1965) for Thorotrast, and for haemoglobin and zymogen by Farquhar & Palade (1963). For other particles not detectable in electron micrographs and to which the cells are finitely permeable, the cellular and intercellular permeabilities must be separately resolved if junction impermeability is to be demonstrated. The outer surface of frog skin is probably sufficiently impermeable to water (MacRobbie & Ussing, 1961) to justify the conclusion of Farquhar & Palade (1965) that the tight junctions between the cornified cells are impermeable to water, although a quantitative argument would strengthen the inference. Similarly, the very low permeability to water of the mucosal surface of the toad bladder makes it unlikely that water passes between the epithelial cells (Peachey & Rasmussen, 1961). The present demonstration that tight junctions in the canal epithelium are impermeable to ions was possible because a lower limit could be set for the intercellular

Fig 32 Section through the wall of an ampullary alveolus The receptor cell (R—P) and nerve terminals (n) that form synapses with them are surrounded by accessory cells (A—A). The apices of R and R debouch on the lumen in which is evident the corpuscular layer (cl). The stalk and two distal parts of the cilium of R are included in the section; the other part of a cilium (c) likely belongs to R. A fibroblast (F) is seen below the basement membrane (bm). The arrows indicate granular agglomerates in the corpuscular zone. *R. lat ta* (Osmium fixed, stained with lead citrate × 6,300)

shunt resistance that was incompatible with the dimensions of the inter cellular space except in the tight junctions It is reasonable to assume that at least in other epithelia the juxtalumenal tight junction or *zonula occludens* has the same property of ionic impermeability However the electron microscopic appearance of the tight junction is not in itself an indication that ions do not normally pass along the space between cells *in vivo* for in the central nervous system Van Harreveld Crowell & Malhotra (1965) have shown that tight junctions and the obliteration of the extracellular space may result from asphyxiation prior to fixation

The significance of the cilia

Merkel (1880 p 45 and Pl 5, Fig 11) described a cilium on the apex of each receptor (flask) cell in the alveolar epithelium of several elasmobranch genera including *Raia* and *Torpedo* but his finding was contradicted by other microscopists after him The present demonstration confirms Merkel's discovery in three species of *Raia* If there are cilia in the ampullae of *Torpedo* Barets & Szabo (1962) may have missed them by not obtaining a section through the apex of a receptor cell In *Plotosus anguillaris* the only teleost species in which ampullae of Lorenzini have been found as many as 8 sensory hairs were noted on each flask cell (Friedrich Frekša 1930)

A consideration of the part that cilia may play in the electrical sensitivity of the ampullae is hardly justified by present knowledge Attention may however be paid to the possibility that their presence indicates a mechanoreceptive function This is called for because cilia have not been reported in two teleost sense organs that may be electroreceptive the small pit organs of *Amiurus* (Mullinger 1964) and the mormyromasts of *Gnathonemus*

Fig 33 The apex of the receptor cell *R* in Fig 32 showing the stalk and two distal parts of the cilium The discontinuity is either apparent because the cilium snakes in and out of the plane of the section or else real because it has broken during fixation The components of the corpuscular layer may be identified the arrow shows a clump of granules *bb* Basal body *t* tight junction *R clavata* (Osmium fixed stained with lead citrate × 26,000)

Fig 34 A portion of the base of the receptor cell *R* seen in Fig 32 A process of the receptor cell deeply invaginates the nerve terminal (*nt*) to form a synapse Synaptic vesicles are lined up along the sides of a pre-synaptic ribbon (*r*) other similar vesicles are seen in the cytoplasm Parts of two accessory cells ([illegible]) insinuate between the receptor cell and the nerve terminal The arrow shows the intercellular space opening above the basement membrane (*bm*) *ly* Presumed lysosomal bodies *m* mitochondrion *n* nucleus *R radiata* (Osmium fixed stained with lead citrate × [illegible],000)


0.5 μ
0.5 μ
m
ly
bb
mb
nt
A_1
A_2
bm
83
34

mens look the same as the material in the lumen which likely represents a solid organic component of the jelly Although their contents are less concentrated the cytoplasmic spaces resemble the vacuoles in the toad bladder that secrete material not dissimilar in appearance the filamentous coating (Peachey & Rasmussen 1961) The fact that they were so rarely seen to communicate with the lumen argues more against their being invaginations cut in cross section than their being secretory vacuoles especially since the lumen in an adult fish would require only topping up to replenish jelly lost at the pore by detrition The arrangement at the 'dense patches suggests that the core is anchored to the epithelium and is not normally extruded A secretory function in these cells does not conflict with their impermeability to ions as there need be no membrane discontinuity when the vacuoles are emptied the impermeability to water of the toad bladder for example does not appear to suffer from the release of the filamentous coating Furthermore the relative abundance of rough surfaced endoplasmic reticulum and the presence of a developed Golgi complex suggests a secretory function for these cells and that the process involved corresponds to the one described for the zymogen granules in the exocrine pancreas (Palade Siekevitz & Caro 1962) It is therefore reasonable that at least the organic constituent of the jelly is secreted by the superficial layer of epithelium in the centrum cap and canal

In the Introduction to Part I reference was made to Lorenzini s (1678) suspicion that the walls of the canal were intended for a hidden function When the canals were found to be electrically insulated it appeared that this function had been discovered Lorenzini himself believing the organs were glands and the jelly their secretion suggested that the walls of the canals could help the ampullae in the filtration of this humour * It would be especially fitting if this suggestion made three centuries ago were also confirmed

* *le pareti dei canali a[illegible] i glo[illegible] alla cribrazione di quello umore*

Summary

I. ELECTRICAL PROPERTIES

1. If the ampullae of Lorenzini in *Raia* are electroreceptors, the long canals by which they communicate with the outside must be adapted to subserve this function. The electrical properties of the ampullary canals were therefore investigated.
2. Theoretical equations were derived for the response of an ampullary canal to a voltage step by solving Kelvin's cable equations with the condition of an open circuit at the ampullary end of the canal.
3. The response to a voltage step of a 10 cm long ampullary canal was recorded with an axial electrode. There was no appreciable spatial decline of the steady state response along the canal when the step was anodal. The response to a cathodal step of more than a few hundred μV was disturbed by oscillatory transients which disappeared when the ampulla was cut off.
4. When a voltage step was applied to a short length of canal that was electrically insulated at its ends, the canal wall behaved like a passive RC network for both anodal and cathodal currents and the average values of mural resistance and capacitance measured in this way were 6.0 MΩ cm^2 and 0.4 μF/cm (10 canals).
5. Mural resistance and capacitance fell to zero when the canal was treated with 1% saponin.
6. The core resistivity measured in 3 canals averaged 31 Ω cm (10° C).
7. The average space constant and mural time constant calculated from the above data were 74 cm and 2.5 sec respectively for a mean canal diameter of 0.12 cm.
8. When the measured values of the electrical constants were inserted into the theoretical equations and numerical solutions computed, satisfactory agreement was obtained with the experimentally recorded response of the ampullary canal.
9. The ampullary canal may be described in electrical terms as an ideal submarine cable (zero leakage conductance and inductance) that is terminated at the receiving end (the ampulla) by an open circuit.

The significance of this for a possible electroreceptive function is discussed

10 While the mural surface resistivity is 30—100 times higher than that of the myelin sheath of nerve, the magnitude of the mural capacity suggests that it derives from only two cell membranes in series

II FINE STRUCTURE

1 The ampullary canals of Lorenzini in *Raia* were examined by light and electron microscopy to identify the structure responsible for the mural insulation and to determine if the relation to it of the receptor cells was compatible with an electroreceptive function

2 The epithelium lining the canal is two cells deep In the electron microscope the cells in the deeper layer are separated laterally by a gap of 100 A or more while the cells of the superficial layer are joined as a rule by tight junctions at the lumenal ends of their side contacts

3 A calculation of the shunt resistance through the space between the deep cells rules them out as forming an effective insulating layer It is further argued that the basement membrane may be excluded and that the superficial layer of cells insulates the canal As a corollary the tight junctions between the superficial cells must be impermeable to ions

4 Nerves do not enter the sensory epithelium directly but *via* a marginal zone that demarcates the epithelium of the canal from that of the alveolus

5 In the epithelium of the ampullary alveoli innervated receptor (flask) cells and un innervated accessory (pyramidal) cells alternate The apex of each receptor cell reaches the lumen and bears a cilium The most commonly observed axonemal pattern was 9 + 1

6 The cells in the alveolar epithelium are joined by tight junctions at the lumenal ends of their side contacts and this arrangement and other evidence indicate that the receptor cells lie across the wall insulation

7 The cuticle reported in the older literature as overlying the sensory epithelium is identified as a layer of aggregated extracellular bodies consisting principally of membrane bound vesicles and fragments of trilaminar membrane

8 It is suggested that the superficial cells of the epithelium of canal and centrum cap from the appearance of their cytoplasmic constituents secrete the organic part of the jelly that fills the lumen

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5-HYDROXYTRYPTAMINE, 5-HYDROXYTRYPTOPHAN DECARBOXYLASE AND MONOAMINE OXIDASE DURING FOETAL AND POSTNATAL DEVELOPMENT IN THE GUINEA-PIG

BY

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PREFACE

This work has been carried out at the Department of Pharmacology University of Helsinki I wish to express my deep gratitude to Professor Armas Vartiainen M D Head of the Department He has placed the research facilities of the Department at my disposal and followed this study through the years with unfailing interest and encouragement

I am very grateful to Associate Professor V J Uuspaa M D for drawing my attention to a 5 hydroxytryptamine study at developmental stages and for reading and criticizing the manuscript To Associate Professor M K Paasonen M D I am very much indebted for his advice and criticism at the beginning of this work For many stimulating discussions my thanks are due to Docent Ulla Hamberg D Sc

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Helsinki October 1965

Anja Tissari

CONTENTS

ABBREVIATIONS

BOL = 2-Bromo-lysergic acid diethylamide
DOPA = 3,4-Dihydroxyphenylalanine
5-HIAA = 5-Hydroxyindoleacetic acid
5-HT = 5-Hydroxytryptamine
5-HTP = 5-Hydroxytryptophan
LSD = Lysergic acid diethylamide
MAO = Monoamine oxidase
S.D.M. = Standard deviation of mean
S.E.M. = Standard error of mean

INTRODUCTION

The occurrence of 5 HT in the brain intestine and blood is common to all vertebrates and since research into 5 HT was started it has been assumed that 5 HT may have a physiological function in these tissues The importance of 5 HT in the brain has been investigated widely and intensively mainly by studying its occurrence and distribution in the brain in detail and by modifying its levels in the brain with a variety of drugs and attempting to correlate changes in 5 HT contents with those in behaviour and other brain functions When investigating the physiological role of 5 HT in the intestine direct application of 5 HT by various routes and stimulation of extrinsic nerves have also been used Despite the great amount of work done the physiological significance of 5 HT is far from being completely known 5 HT during developmental stages has been neglected for a relatively long time A knowledge of its occurrence during development however could well throw light on its significance in the tissues in which it is assumed to play a role In addition information on its appearance and development in various tissues could elucidate the yet unsolved question of its origin in different sites in the body

The enzymes associated with 5 HT — 5 HTP decarboxylase in its formation and MAO in its inactivation — are nonspecific They also play a key role in the formation and metabolism of catechol amines The question of other physiologic substrates of 5 HTP decarboxylase is still open The substrate spectrum of MAO includes a large number of aliphatic and aralkyl amines Data on the development of 5 HTP decarboxylase and MAO activities in their typical sites during maturation could aid in understanding the physiological role of these enzymes These were the aims of the present work

The development of 5 HT contents in the brain intestine and blood during maturation has been examined. A simultaneous study has been made of 5 HTP decarboxylase and MAO activities In addition to the brain and intestine their development has been studied in two other rich sources the kidney and liver These subjects were investigated in the guinea pig from an early stage of foetal development up to adulthood or until the adult levels were attained An attempt has been made to explain the low tissue 5 HT levels found during development by means of experiments in which the precursor amino acid, 5 HTP was administered The changes in 5 HT contents and 5 HTP decarboxylase and MAO activities have been correlated with the functional and morphological development of the organs in question as far as data on these are available in the literature

In 1958, when the studies described in this paper were begun only two reports on 5 HT contents during the developmental period could be found in the literature (Faustini 1955 Parratt and West 1957) Reports on the development of the associated enzymes were also lacking except a few data on MAO activities in human autopsy material (Birkhauser 1940 Zeller et al 1940 Epps 1945) In the course of this work several other reports have appeared principally with regard only to the contents of 5 HT and the associated enzymes in the brain during development

The present study is preceded by a brief review of the data now available on the occurrence and physiological significance of 5 HT 5 HTP decarboxylase and MAO in the tissues in question This chapter does not deal with papers concerning these subjects during development instead they are referred to in the discussion on the present results

This paper provides a complete survey of earlier short reports (Tissari 1960 1963, 1964 1965)

REVIEW OF THE LITERATURE

5-HYDROXYTRYPTAMINE IN THE BRAIN, GASTROINTESTINAL TRACT AND BLOOD

5-Hydroxytryptamine in the Brain

Occurrence and Distribution

The occurrence of 5-HT in the brain was first demonstrated by Twarog and Page (1953). Amin et al. (1954) and Zeller and Schlosser (1954) found 5-HT in the brain while studying the distribution of substance P there. These studies were performed by biological methods. The occurrence of 5-HT in the brain was confirmed fluorimetrically by Bogdanski et al. (1956). Since then 5-HT has been found in the brain of all mammals and of all other vertebrates studied.

Amin et al.'s investigation (1954) concerned the distribution of 5-HT in the brain. They found it in large quantities in the mesencephalon and diencephalon, particularly in the hypothalamus. There was no 5-HT in the cerebellum, but an abundance of it in the area postrema. In the telencephalon Paasonen et al. (1957) discovered that the limbic system — phylogenetically the oldest part of the cortex — contained 5-HT and that its contents were greatest in the area of the trigonum olfactorium; in the neocortex they were small. 5-HT was also present in the caudate nucleus. Bogdanski et al. (1957) confirmed the distribution of 5-HT in the brain of several test animals and of man fluorimetrically. Costa and Aprison (1958) studied the distribution of human brain 5-HT in detail; their results fully resembled those obtained from test animals. Later it was found that the pineal body contained large quantities of 5-HT (Giarman and Day 1958, Giarman and Freedman 1960); in the rat the content was as high as 57—73 µg/g (Bertler et al. 1963).

Using a fluorescence microscope, Carlsson et al. (1962) detected a fluorescence typical of 5-HT in the regions surrounding the preoptic area and in the amygdala of the rat brain; it appeared in smooth fibres — probably nerve fibres. After MAO inhibition 5-HT fluorescence was also visible in the nerve cells and fibres in the posterior hypothalamus and in the pons of the mouse. In rat brain Dahlström and Fuxe (1964) found cell bodies containing 5-HT in the raphe nuclei of the medulla oblongata, pons and mesencephalon, from which ascending and descending nerve processes led off. The cell bodies displayed fluorescence only after MAO inhibition. In the pineal body 5-HT was stored in the parenchyma cells and sympathetic pineal nerves of the rat and mouse, but only in the pineal nerves of the guinea pig and dog (Bertler et al. 1963).

Subcellular distribution. Walaszek and Abood (1959) found that the mitochondrial fraction contained 63 %, the nuclear fraction 22 %, and the supernatant 12 % of the total 5-HT in rat

brain homogenate there was none in the microsomal fraction When the particles were separated from the homogenate directly by a single high speed centrifuging the free and bound 5-HT were obtained in the following proportions free 24 % bound 76 % (Giarman and Schanberg 1958) 26 and 74 % (Michaelson and Whittaker 1963) The binding of 5 HT to the particles is very labile when brain homogenate is split into several particulate fractions by differential centrifugation the 5-HT quantity remaining in the supernatant is considerably greater — as much as 50 % (Giarman and Schanberg 1958 Whittaker 1959)

Walaszek and Abood's (1959) discovery that of the primary fractions of brain homogenate the mitochondrial fraction contains the most 5 HT has been confirmed by several investigators who further divided it into subfractions of myelin nerve-ending particles and mitochondria. They found that the highest 5-HT content was in the nerve-ending particle subfraction — Whittaker in the guinea-pig (1959) Potter and Axelrod (1962) and Carlini and Green (1963) in the rat. Similarly Laverty et al (1963) noted that in the dog particulate 5-HT was associated mainly with the primary mitochondrial fraction of the caudate nucleus and hypothalamus Whittaker's group (Michaelson et al. 1963) further studied 5-HT in the nerve-ending particles Upon suspension in water the cytoplasm and synaptic vesicles were released, and the vesicles and other particles were separated by density gradient centrifugation. By treating each subfraction with water they were able to separate the free 5-HT contained in the particles Thus they found that some 70 % of the 5-HT in the primary mitochondrial fraction could be released in this way — more from the smaller than from the larger particles Bound 5-HT was distributed between all the subfractions slightly more being present in the region of the disrupted nerve endings than in the synaptic vesicle subfraction. Carlini and Green (1963) detected the largest quantities of particulate 5-HT in the two fractions corresponding to the nerve-ending particles and synaptic vesicles

Besides the nerve-ending particles Ryall (1962) observed great 5-HT activity in the microsomal fraction. Zieher and de Robertis (1963) found that 5-HT was divided equally between the nerve endings and microsomes A synaptic vesicle fraction prepared after hyposmotic shock contained no 5-HT

Physiological Significance

Since LSD which in small doses causes mental disturbances was found to antagonize the action of 5-HT on smooth muscle Gaddum (1954) and Woolley and Shaw (1954) assumed that the effect of LSD on the brain was related to its 5-HT inhibition and thought that 5-HT might play a rôle in brain function.

Owing to the poor passage of 5-HT through the blood brain barrier (Udenfriend et al. 1957b) its effects on brain function have mainly been studied indirectly using drugs that change the 5-HT content of the brain. Brodie Pletscher and Shore (1955) showed that reserpine liberates bound brain 5-HT and that the sedation and low brain 5-HT level persists for a long time after all the extractable reserpine has disappeared from the brain. They assumed that reserpine acts through free 5-HT and that 5-HT may play a rôle in brain function as a neurohumoral agent Then in 1957 Brodie and Shore (Brodie and Shore 1957 Brodie 1958) presented the hypothesis that 5-HT acts as a chemical mediator in the trophotropic division of Hess's (1954) subcortical system, and noradrenaline (Vogt 1954) in its ergotropic division Since reserpine set off the same kind of effect as stimulation of the trophotropic system Brodie and Shore suggested that reserpine acts by causing a continual release of brain 5-HT and that the free 5-HT stimulates synapses of the trophotropic system in the diencephalon. Later on, Brodie's group produced additional evidence that the sedation produced by reserpine is caused by free 5-HT and not by the release

of noradrenaline in the brain (Holzbauer and Vogt 1956) When reserpine was administered to cold-exposed rats the brain noradrenaline was depleted but not the brain 5-HT and no sedation was induced In small doses too a reserpine analogue Su 5171 depleted half of the noradrenaline but none of the 5 HT in the rabbit brain and no sedation occurred. Large doses liberated about 70 % of both amines and caused sedation (Brodie et al 1960) α Methyl m-tyrosine produced a slight transient reduction of 5-HT nor drenaline was almost entirely depleted but there was no sedation In large doses α methyl m tyrosine depleted 35% of the 5-HT without sedation The authors concluded that depletion of catechol amines in the brain does not produce sedation but that the effect of reserpine is related to changes in the storage of 5-HT in the brain (Gessa et al 1962 Costa et al. 1962)

Carlsson et al (1957) investigated the extent to which the central effect of reserpine correlated to changes in the catechol amines and/or 5-HT Sedation produced by reserpine in the mouse was antagonized by DOPA but not by 5-HTP Iproniazid pretreatment diminished the DOPA dosage required Carlsson et al (1960) discovered that nialamide treatment of reserpine treated mice increased brain 5-HT and caused tremors and convulsions but did not restore alertness 5-HTP administration had a similar effect. They concluded that lack of catechol amines explains at least part of the tranquillizing effect of reserpine Alertness is correlated to the catechol amines rather than the 5-HT

Carlsson (1964) has recently suggested that the effect of reserpine is due to a blockage of the storage of monoamines when their labile fraction disappears nerve transmission ceases Thus the pharmacological effects of reserpine are correlated to the storage capacity rather than to the tissue amine levels 5-HT neurones may play a part in regulating the motor functions of the brain because 5-HTP causes tremors convulsions and hyperextension of the limbs 5-HTP is unable to antagonize akinesia produced by reserpine In suitable doses however DOPA restores motility to normal and partly restores even more complex functions such as conditioned avoidance response in reserpine treated animals Thus 5-HT dopamine and noradrenaline seem to act mainly as excitatory transmitters in the brain.

Monoamines in the spinal cord have also been studied at the same laboratory and the results indicate that 5-HT meets the criteria of a neurohumoral transmitter in descending pathways of the spinal cord originating in the brain The major evidence is as follows from dissected spinal cord of nialamide pretreated mice and frogs 5-HT was liberated after electrical stimulation and 5-HT synthesis was augmented (Andén et al 1964a) Administration of 5-HTP to spinal cats caused excitation of motoneurones The motoneurone excitation in decerebrated cats produced by electric stimulation of the caudal medulla disappeared after reserpine treatment (Andén et al. 1964b)

5 Hydroxytryptamine in the Gastrointestinal Tract

Occurrence and Distribution

As early in 1933 Vialli and Erspamer demonstrated histochemically the presence of a di o polyphenol derivative in the granules of the granulated cells in the gastrointestinal mucosa In 1937 they detected it chemically in acetone and alcohol extracts of the same tissues and in 1940 Erspamer stated that the same extracts were biologically active he called the active substance enteramine In 1953 Dalgliesh et al. identified the enteramine in extracts of mammalian small intestine as 5-HT Feldberg and Toh (1953) studied the distribution of 5-HT in the gastrointestinal tract of the dog and found that its content was greatest in the pylorus and smallest in the oesophagus and while in the other parts of the gastrointestinal tract th distribution was

even All the 5 HT was found in the mucosa almost none in the muscularis externa In the rabbit the gastric fundus contained more 5 HT than the pylorus Erspamer (1953 1954b) charted the distribution of 5-HT in the gastrointestinal tract of numerous species of vertebrates and some invertebrates The distribution between various parts of the gastrointestinal tract differed among species In the dog mouse and hedgehog the 5 HT content was largest in the stomach In the guinea pig chicken and rabbit the highest concentration was in the small intestine The greatest content in the rat was found in the large intestine The same held good for the cat in which the 5 HT content in the gastrointestinal tract was extremely small In the horse and ox too the colon had the highest content (Erspamer 1961b) In man Erspamer (1953 1954b) found the highest content in the duodenum and colon in the stomach there was more 5 HT in the pylorus than in the fundus According to Resnick and Gray (1961) the 5 HT content was significantly smaller in the human colon than in the small intestine and there were no differences between the various parts of the stomach 5-HT was present in the gastrointestinal tract of reptiles amphibians and fishes (Erspamer 1953 1954b Bogdanski et al 1963) though it was probably absent in that of the cyclostomata the most primitive species of fish (Erspamer 1961b) In these classes of vertebrates the 5-HT contents were similar to those of mammals (Bogdanski et al 1963)

Histologists have long held differing views on the nature of the specific substance of enterochromaffin cells it was earlier regarded as some kind of diphenol derivative More recently Glenner and Lillie (1957) and Lillie (1957) using histochemical methods specific for indole derivatives failed to detect any 5 HT in the enterochromaffin cells of the gastrointestinal tract Using a variety of histological methods Barter and Pearse (1953 1955) concluded that the enterochromaffin granules contained 5 HT Benditt and Wong (1957) came to the same conclusion after using a number of histochemical techniques On the basis of histochemical and certain indirect evidence Erspamer (1961b) considered it highly probable that all or a large part of the gastrointestinal 5 HT is located in the enterochromaffin cells Norberg (1964) studied different parts of the rat and cat intestine using a fluorescence microscope and discovered cells with an intense yellow fluorescence typical of 5 HT in the mucosal epithelium These cells were no doubt identical with the enterochromaffin cells containing 5 HT

Subcellular distribution When Baker (1958) fractionated homogenates of dog duodenal mucosa by centrifuging she found that most of the 5 HT was recovered in the mitochondrial fraction It was strongly bound to the granules and was not released even when recentrifuged This method did not make clear how much of the total 5 HT of the mucosa was in the granules In the intestinal mucosa of the ox Bertler et al (1960) found that the mitochondrial fraction contained about half of the low speed supernatant 5 HT Baker (1959) further studied the mitochondrial fractions by ultracentrifuging them in hypertonic sucrose solution and found that the 5 HT was recovered in heavier granules than the mitochondrial enzymes were In the same laboratory Prusoff (1960) confirmed Baker s discovery He found adenosine triphosphate in both the mitochondrial fraction and the 5 HT fraction and in the latter the 5 HT adenosine triphosphate molar ratio was nearly 3 — approximating the catechol amine adenosine triphosphate molar ratio of 4 in the adrenal medulla

5 Hydroxytryptamine and Intestinal Motility

Effect of 5 HT on intestinal motility When administered to the serosa of the guinea pig ileum in vitro 5 HT stopped peristalsis (Kosterlitz and Robinson 1957 Ginzel 1957) but not when administered to the serosa of the rabbit jejunum (Bulbring and Lin 1958) 5 HT (10^{-9}—10^{-5}) infused intraluminally into the small intestine of the guinea pig and rabbit stimulated peristalsis and reduced the pressure threshold (Bulbring and Lin 1958) Bulbring and Crema (1958) went

on to study the site of 5-HT action in the peristaltic reflex and concluded that 5-HT stimulated the sensory receptors of the mucosa and sensitized the muscle to acetylcholine. In small doses it stimulated the ganglia: serosal 5-HT restored peristalsis that had been stopped by hexamethonium and atropine. In large doses it blocked the ganglia, and so serosal 5-HT stopped peristalsis. Lembeck (1958a) considered that the mode of action of 5-HT is to sensitize the afferent structures of the intestine to distension, and regarded 5-HT mainly as a local tissue hormone.

On the subject of 5-HT receptors in the longitudinal muscle of the guinea-pig ileum, Rocha e Silva et al. (1953) suggested that 5-HT stimulated the postganglionic parasympathetic nerve fibres. Gaddum and Hameed (1954) assumed that 5-HT stimulated receptors in the intramural ganglion cells that differed from the ganglion receptors stimulated by acetylcholine and nicotine. Later on, Gaddum and Picarelli (1957) found two types of 5-HT receptors in the guinea-pig ileum — M and D receptors. The former could be blocked by morphine and were located in nervous tissue, probably ganglia; the latter could be blocked by phenoxybenzamine (dibenzyline) and were in the muscle. Day and Vane (1963) demonstrated that morphine was only a partial antagonist of 5-HT receptors in nervous tissue, and phenoxybenzamine was non-specific, affecting both types of receptors. They found that in the guinea-pig ileum, 5-HT acted mainly on the nerve receptors and that its effect on the muscle receptors under normal conditions was insignificant. According to Brownlee and Johnson (1963) 5-HT only affected specific receptors in the intramural cholinergic ganglion cells and it activated a nerve pathway independent of that activated by dimethylphenylpiperazinium (Johnson 1964).

In isolated guinea-pig and rabbit colons, intraluminal 5-HT stimulated peristalsis; long infusion was followed by inhibition. Serosal 5-HT always stimulated peristalsis in the rabbit, and in the guinea-pig, contractions were followed by inhibition, but in both it facilitated the response to parasympathetic extrinsic pelvic nerve stimulation (Lee 1960). Isolated rat and mouse stomachs were very sensitive (10^{-10}) to contraction by serosal 5-HT but guinea-pig and cat stomachs reacted poorly even to large doses. Intraluminal 5-HT caused no contractions in the guinea-pig stomach. In rat and mouse stomachs 5-HT acted mainly on the muscle receptors since its action was affected neither by morphine nor by ganglion-blocking agents (Paton and Vane 1963).

5-HT also contracted the intestine of teleosts and elasmobranchs in vitro. Some species of teleosts were very sensitive in their reactions (e.g. [illegible] and Ostlund 1957).

Bülbring and Crema (1958b) studied the effect of 5-HT on peristalsis in the guinea-pig small intestine also using jejunal loops in situ. Contrary to the situation in vitro, 5-HT administered in vivo intraluminally or into the circulation either inhibited peristalsis, as also did large doses of 5-HTP. Stimulation was best produced by small intra-arterial 5-HT or 5-HTP infusions or intraluminal 5-HTP infusion at low intraluminal pressure. The reason for the discrepancy was that the local production of 5-HT in vitro had declined, while in vivo it continued undiminished; the effect of 5-HT on peristalsis depends on its local concentration in the region of the mucosal sensory receptors and possibly of other structures associated with the peristaltic reflex. Below a certain limit, it stimulates peristalsis; above that, it inhibits it.

Considerable research has been made into the effect of 5-HT on the motility of the gastrointestinal tract in the intact organism. In man, intravenous 5-HT (Hendrix et al. 1957) and 5-HTP (Haverback and Davidson 1958) raised the motility of the jejunum in doses that did not affect other functions. Atropine inhibited the effect of 5-HT; BOL did so partly, but hexamethonium not at all. 5-HT and 5-HTP administered intraluminally did not affect intestinal motility. Similar doses of atropine did not inhibit the effect of 5-HT in the experiments of Daniel et al. (1961) with human subjects. In the dog, a small dose of intravenous 5-HT raised the motility of the small intestine ([illegible] et al. [illegible], Haverback et al. 1957). Atropine and hexamethonium

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strong BOL reduced the peristalsis but did not abolish it. In addition reserpine depleted the catechol amines in the intestine and in a reserpine-treated guinea-pig intestine sympathetic nerve stimulation caused contraction instead of inhibition (Gillespie and Mackenna 1961). Reserpine also raised the acetylcholine content of the intestine (Malhotra and Das 1962).

Boullin (1964) brought about selective depletion of intestinal 5-HT in rats with a tryptophan-deficient diet. Peristalsis in vitro and in situ was normal in these rats and 5-HT stimulated the peristaltic reflex in the same way as in rats with a normal intestinal 5-HT content. He concluded that 5-HT was not essential for peristalsis as a sensory stimulant or in any other way.

Occurrence of 5-Hydroxytryptamine in the Blood

Rapport (1949) identified the ox serum vasoconstrictor and Rand and Reid (1951) the vasoconstrictor of ox platelets as 5-HT. Erspamer and Faustini (1953) charted the 5-HT contents of the serum and spleen in a large number of vertebrates. They found great differences between both species and individuals. The 5-HT contents of the serum and spleen displayed a mutual correlation, but none with the content of the intestine. Among mammals the cat, rabbit and goat had the highest 5-HT content in the serum, and man, dog and guinea-pig the lowest. Similarly the highest ratio of serum 5-HT to total 5-HT was found in the cat (70.7) and rabbit (33.2) while in the guinea-pig it was only 4.6 . Udenfriend and Weissbach (1954) found a similar occurrence in the whole blood of different species. Humphrey and Jaques (1954) studied the 5-HT content of the platelets in several mammals: in the cat it was low and in other species the ratios were as above.

All the blood 5-HT is in the platelets, none at all in the plasma (Udenfriend and Weissbach 1954; Humphrey and Jaques 1954).

When Baker et al. (1959) centrifuged platelet homogenates over a density gradient, they found most of the 5-HT and adenosine triphosphate in a fraction that was denser than 2.0 M sucrose; the density of intact platelets was equivalent to 1.6 M sucrose. They concluded that the bulk of the 5-HT in the platelets is concentrated in cytoplasmic granules in which it is associated with adenosine triphosphate. On the contrary, Hughes and Brodie (1959) observed that after platelets had been homogenized in isotonic sucrose the 5-HT was ultrafilterable and so could not be granular bound.

5-HYDROXYTRYPTOPHAN DECARBOXYLASE

Occurrence and Distribution

Udenfriend et al. (1953) were the first to demonstrate 5-HTP decarboxylase activity in the kidney of the dog and the guinea-pig. Clark et al. (1954) further investigated its occurrence and properties and found that the kidney and liver of all the mammals examined contained this enzyme; it was also detected in several other tissues, but not in platelets. Gaddum and Giarman (1956) studied its occurrence and distribution in several species of mammal. The greatest activity in all the species was noted in the kidney. The liver and intestine also contained considerable quantities, but none was found in the plasma, platelets or bone marrow. Similar findings were made by West (1958) in seven mammal species. Recently Klingman et al. (1964) have investigated the distribution of DOPA decarboxylase in the gastrointestinal tract of the rat. They found the activity to be greatest in the small intestine, decreasing aborally; activity was sign-

did not inhibit the effect of 5 HT but BOL did so wholly or partly (Haverback and Davidson 1958 Daniel et al 1961)

By injecting 0 17 mg/kg of 5 HT subcutaneously to rats Lembeck (1958b) raised the number of defecations to almost double With corresponding injections of 5 HT and 5 HTP Fish et al (1959) found that the stomach emptied slightly faster than normal but the evacuation of the small intestine was strongly inhibited In the mouse the 5 HT and 5 HTP doses required to increase the number of faeces passed were 1 mg/kg and 10 mg/kg administered subcutaneously (Brittain and Collier 1958 Erspamer et al 1960) 5 HT had no effect on the intestinal transit of a test meal (Brittain and Collier 1958)

To sum up intestinal motility is stimulated by intra arterial doses of 5 HT in the order of physiological release 0 05 — 0 1 μg/kg in the guinea pig (Bulbring and Crema 1959b) as little as 0 001 μg/kg in the dog injected into the appropriate mesenteric artery (Sleisenger et al 1959) and 1—2 μg/kg injected into the thoracic artery in the cat (Lembeck 1958b)

Release of 5 HT from the intestine caused by motility Bulbring and Lin (1958) found in the small intestine of the rabbit and guinea pig in vitro and Bulbring and Crema (1959a) in in situ loops of the guinea pig ileum that 5 HT was released continuously into the lumen of the intestine the amount of 5 HT released rose about 3 fold in vitro and 8 fold in vivo when the intraluminal pressure was increased above the peristaltic threshold in the latter case it amounted to roughly 0 03 μg/min/5—7 cm loops When peristalsis was blocked by atropine or hexamethonium some irregular movement continued and the change in 5 HT release caused by the increase of pressure diminished but did not cease In vivo the intraluminal pressure was kept high for several hours without diminishing the 5 HT release

The quantity of 5 HT released in isolated aboral sections of guinea pig and rabbit distal colon was only a fraction of that released in the oral section and in the ileum (Lee 1960) There was no 5 HT response to a rise in the intraluminal pressure in the oral section of the distal colon when peristalsis was entirely abolished by anoxia or adrenaline Stimulation of the parasympathetic pelvic nerve increased peristalsis and 5 HT release and stimulation of the sympathetic nerve reduced them Lee concluded that 5 HT release is regulated by an intrinsic mechanism associated with peristalsis and not by the extrinsic nerves

Paton and Vane (1963) investigated the substances released by nerve stimulation from the isolated stomach of several mammals 5 HT was detected occasionally even at rest When stomach tone was raised by nerve stimulation LSD anticholinesterases or mechanical distension 5 HT release increased many times the highest value being found in the guinea pig — 2—12 ng/min/stomach Thus 5 HT release appears to depend on the mechanical deforming effect of the contracted muscle on the cells in the wall that contain 5 HT

When intestinal motility was increased the 5 HT content in the venous blood from the segment in question rose in the rabbit (Karki et al 1960) In man Adams (1960) and Bojs (1961) discovered an increase even in the systemic blood level

The intestinal 5 HT content was not reduced either in vitro (Bulbring and Lin 1958) or in vivo (Pletscher et al 1955 Paasonen et al 1960) by raised motility The quantity of enterochromaffin cells detectable histochemically diminished in the rat duodenum when the intraluminal pressure was raised (Cole et al 1961) but in Crohn s disease and intestinal tuberculosis in which the intestine is changed into a rigid tube the number and size of the enterochromaffin cells and the quantity of granules in them increased (Pearse 1958)

Peristalsis after depletion of intestinal 5 HT That diarrhoea is produced by reserpine in man (Vakil 1954) and in test animals (Pletscher et al 1955) is well known After intensive reserpine treatment of guinea pigs which depleted the 5 HT content of the intestinal mucosa to as little as 1 Bulbring and Crema (1959b) found that peristalsis in in situ intestinal loops continued

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ficantly less in the pyloric antrum and caecum than in the small intestine On the other hand the 5 HT and noradrenaline content discovered in the pyloric antrum and caecum were extremely high 5 HTP decarboxylase activity has been observed in the mast cells of the rat (Lagunoff and Benditt 1959) and in mouse mastocytoma (Hagen et al 1960)

Gaddum and Giarman (1956) examined the distribution of 5 HTP decarboxylase in the brain of several mammal species They found a correlation between the 5-HT and 5 HTP decarboxylase contents everywhere except in the area postrema in which there was a relatively high concentration of 5-HT but no decarboxylase activity High 5-HTP decarboxylase activity was detected in the nucleus caudatus and hypothalamus but there was little activity in the cerebral and cerebellar cortices Bogdanski et al (1957) studied the distribution of 5 HT 5-HTP decarboxylase and MAO in the brain of the dog and the cat In general they found a similar distribution of 5 HT and 5-HTP decarboxylase the exceptions were the amygdala and pyriform cortex in which there was abundant 5 HT but low 5 HTP decarboxylase activity and the caudate nucleus where the decarboxylase activity was high and the 5-HT content only average The highest 5 HTP decarboxylase activity observed was in the region of the neostriatum and brain stem Kuntzman et al (1961) investigated the distribution of 5 HTP decarboxylase in the cat brain in detail. The greatest activity was found in the reticular formation the hypothalamus the basal and intralaminar parts of the thalamus the olfactory tubercle septum and amygdala in the rhinencephalon and the caudate nucleus

Subcellular distribution Giarman (1956) ultracentrifuged homogenates of the kidney liver and gastrointestinal tract and noted 5 HTP decarboxylase activity only in the non particulate fraction The same finding was made by Bogdanski et al (1957) in the brain and Hagen et al (1960) in mouse mastocytoma cells

Physiological Significance

When Clark et al (1954) decarboxylated DL 5 HTP by 5 HTP decarboxylase it yielded only half the calculated amount of 5 HT and so they assumed that only the L isomer could serve as a substrate Using isolated L-5 HTP D 5 HTP and DL 5 HTP Freter et al (1957) confirmed the optical specificity of 5-HTP decarboxylase In man however excretion of 5 HT and 5 HIAA have been found to increase after D 5 HTP administration probably owing to the conversion of D 5 HTP to L-5 HTP in vivo (Oates and Sjoerdsma 1961)

Westermann et al (1958) found that the distribution of DOPA and 5 HTP decarboxylases was the same in several tissues and in many species α methyl DOPA inhibited both enzymes They assumed that DOPA and 5 HTP decarboxylase were identical Bertler and Rosengren (1959) came to the same conclusion after discovering that DOPA decarboxylase of kidney and brain extracts was competitively inhibited by 5-HTP and vice versa and also that the distribution of both enzymes in the brain was the same Yuwiller et al (1959) noted that 5-HTP and and DOPA competed for decarboxylation and that the decarboxylation of both was blocked by the same inhibitors When Werle and Aures (1959) and Rosengren (1960a) purified a DOPA decarboxylase preparation they found the same activity ratios for DOPA and 5-HTP as in the crude extracts This strengthened the assumption that they were one and the same enzyme

Findings on other substrates of 5 HTP decarboxylase differ Udenfriend et al (1960) and Lovenberg et al (1962) noted that this single enzyme decarboxylated all the naturally occurring aromatic L-amino acids It also decarboxylated unnatural aromatic amino acids such as aromatic α methylamino acids 50- to 100 fold purification of the enzyme caused no dissociation of the activities The natural L amino acids that it decarboxylated were DOPA 5 HTP tryptophan

tyrosine phenylalanine and histidine The affinity of amino acids other than DOPA and 5 HTP for the enzyme was quite low Histidine was also decarboxylated by another enzyme specific I histidine decarboxylase and this enzyme probably plays a major role in generating histamine for physiological processes (Weissbach et al 1961b) Rosengren (1960a) too discovered histidine decarboxylase in the rabbit kidney and this was identical with DOPA decarboxylase and differed from specific histidine decarboxylase Parallel changes in DOPA decarboxylase 5-HTP decarboxylase and non specific histidine decarboxylase have recently been shown to occur in the rat liver when rats are fed with hepatic carcinogen diethylnitrosamine (Reid et al. 1963) or a tryptophan-deficient diet (Reid and Shepherd 1964) The results suggest that these three activities are due to a single enzyme, a non specific aromatic amino acid decarboxylase

In the above mentioned studies the amines formed were separated from unreacted substrates by means of ion-exchangers and determined spectrophotometrically or spectrofluorimetrically Hagen (1962) measured the enzyme activity with the aid of the CO_2 formed or using ^{14}C labelled substrates with the aid of the amines formed detected autoradiographically He found that DOPA decarboxylase prepared from ox adrenal medulla human phaeochromocytoma and human argentaffinoma did not decarboxylate tyrosine tryptophan or histidine. Awapara et al (1962) also discovered that none of these amino acids were decarboxylated by a purified DOPA decarboxyla e preparation of the rat liver Recently Awapara et al. (1964) used ^{14}C labelled DOPA tyrosine and phenylalanine as substrates and purified guinea pig kidney preparation as the enzyme source and measured the $^{14}CO_2$ formed They were unable to demonstrate the decarboxylation of tyrosine and phenylalanine with certainty

Amines of all the dietary amino acids are excreted in the urine (Sjoerdsma et al. 1959) and they have been regarded as originating in a reaction catalysed by tissue DOPA decarboxylase (Oates et al 1960) Awapara et al (1964) suggested that these aromatic amines were more likely to originate in decarboxylation caused by bacteria in the intestine

MONOAMINE OXIDASE

Occurrence and Distribution

The presence of MAO in the brain was first demonstrated by Pugh and Quastel (1937) using aliphatic amines as substrates Birkhauser (1940) studied the occurrence of MAO in several sites in the human brain and noted that its content was roughly similar in different sites it was highest in the thalamus and lowest in the cortex but the ratio between them was only 2 1 Bogdanski Weissbach and Udenfriend (1957) studied the distribution of MAO activity in the dog brain using 5-HT as substrate and came to the same conclusion. the activity was very similar in different parts of the brain except in the hypothalamus where it was some $1^1/_2$ times greater and in the white matter in which it wa roughly half the level found in other parts of the brain.

Using a histochemical method Arioka and Tanimukai (1957) investigated the distribution of MAO in the diencephalon of the mouse and Shimizu et al (1959) in the brain of four rodent species The highest activity was discovered in the hypothalamus and the blood brain barrier In the somatic areas of the brain MAO activity was low On the basis of the distribution Shimizu et al. concluded that MAO is associated with the metabolism of the visceral areas of the brain and not only with the function of the adrenergic neurones

Blaschko et al. (1937b) studied MAO activity in numerous tissues of several species both vertebrate and invertebrate using different amines as substrates They found that in the guinea

pig rat and pig MAO activity was highest in the liver In the guinea pig this was followed by the intestine kidney and brain remarkable differences in activity being noted between each In the rat, it was higher in the lungs than in the kidney Bhagvat et al (1939) further studied the distribution of MAO in several mammal species and noted similarly that the liver kidney and intestine were the richest sources of MAO though some activity was found in most of the tissues examined. There was considerable activity in the uterus and in some species also in the spleen. It was low in the heart and in skeletal muscle. According to Klingman et al. (1964) the MAO content in the different parts of the gastrointestinal tract of the rat was more uniform than either DOPA decarboxylase or the 5-HT and noradrenaline contents They found that the highest activity was in the jejunum, decreasing from there in both directions

MAO activity in human tissues has been studied by Langemann (1944) and Levine and Sjoerdsma (1962) The distribution differed from that of several other species in that the MAO activity in the human heart was high while in most other species it was low In man as in guinea pig and rat, MAO activity was highest in the mucosa of the jejunum (Levine and Sjoerdsma 1962)

Blaschko and Hellmann (1953) and Koelle and de Valk Jr (1954) studied the cellular distribution of MAO in several mammal species by histochemical methods In the liver the activity was localized in the parenchyma cells and in the kidney in the proximal tubules The glomeruli were inactive. Koelle and de Valk Jr found the MAO activity in the ileum and stomach of the cat to be greatest in the epithelial cells of the mucosa moderate in the ganglion cells and fibres of Meissner s and Auerbach s plexuses the walls of the arteries and the muscularis mucosae and practically non-existent in the muscularis externa. The converse distribution of MAO was found by Blaschko (1952b) in the small intestine of the sheep the muscular coat of which contained more of the enzyme than the mucosa did

Plasma does not contain MAO according to Blaschko et al (1959) and Levine and Sjoerdsma (1962) Despite the high MAO content of the human placenta (Luschinsky and Singher 1948) none has been found in the plasma even during pregnancy (Werle and Pechmann 1949) Using an extremely sensitive radioisotope assay method, Otsuka and Kobayashi (1964) recently demonstrated very weak MAO activity in human plasma. In addition to MAO catalase was present but aldehyde oxidase was lacking Waalkes and Coburn (1958) found MAO in the erythrocytes of the rabbit. Paasonen et al. (1964) have lately discovered slight MAO activity in human blood platelets. Similarly when Pletscher and Bartholini (1964) incubated rabbit blood platelets with reserpine liberated 5-HT appeared in the incubation solution mostly as 5 HIAA and 5-hydroxytryptophol

Subcellular distribution Cotzias and Dole (1951) studied the distribution of MAO in subcellular fractions of the rat liver and noted that it was probably located entirely in mitochondria. In the same tissue Hawkins (1952) found $^4/_5$ of the MAO activity in the mitochondrial fraction and the rest in the microsomal fraction the mitochondrial contamination of which was not known Oswald and Strittmatter (1963) described a dual subcellular distribution of MAO in the rat liver in addition to the approximately 75 % in the mitochondria there was a smaller but significant percentage in the microsomal fraction but it was not due to mitochondrial contamination. Weiner (1960) and Rodriguez de Lores Arnaiz and De Robertis (1962) found that the brain MAO was entirely in the mitochondrial fraction. When the primary mitochondrial fraction was split into subfractions 60 7 % of the MAO activity was found in the free mitochondrial subfraction and 36 7 % to that of the non-cholinergic nerve endings When it was further centrifuged after osmotic shock treatment, the entire MAO activity was found in the subfraction containing free mitochondria and nerve endings The subfraction containing the synaptic vesicles was inactive Rodriguez de Lores Arnaiz and De Robertis 1962)

Weissbach et al. (1957) demonstrated soluble MAO in tissue homogenates prepared in water. It was found in considerable quantities in the guinea pig liver and was also detected in the guinea pig kidney and rat liver. Oswald and Strittmatter (1963) too discovered some 10 % of soluble MAO after lytic treatment of total particulate preparations of the guinea-pig liver.

Physiological Significance

In 1929 Hare described an enzyme in mammal tissues that oxidized tyramine. Blaschko et al. (1937a) described adrenaline oxidase and Pugh and Quastel aliphatic amine oxidase in 1937. Kohn (1937) and Blaschko et al. (1937b) demonstrated that it was the same enzyme — amine oxidase — that catalysed the same oxidation reaction of a large number of amines. Bradley et al. (1950) found that 5-HT was inactivated by MAO and its rate of oxidation was similar to that of tyramine and of tryptamine (Blaschko 1952a). Histamine was also a weak substrate of MAO and the activity of this enzyme towards it amounted to only 0.7 % of that towards tyramine (Zeller et al. 1956).

Significance of MAO in the metabolism of 5-HT. The main pathway in the metabolism of 5-HT is oxidative deamination catalysed by MAO. When MAO inhibitors were administered in vivo the 5-HT content of the brain rose in all the mammal species studied — rodents (Pletscher 1956b, Udenfriend et al. 1957a), cat and dog (Spector et al. 1960). In the intestine, however, the 5-HT content rose very little (Pletscher 1956a) or not at all (Spector et al. 1960). After continued iproniazid treatment the 5-HT content of the blood increased strongly in man and rabbit (Shore et al. 1958, Pletscher and Bernstein 1958).

The pharmacological effects of 5-HT in vivo were prolonged by iproniazid treatment in the rat (Sjoerdsma et al. 1955). In mice treated with a MAO inhibitor the metabolism of a small 5-HT dose was retarded (Axelrod and Inscoe 1963). The disappearance of a large dose of 5-HT was not affected (Udenfriend et al. 1957a), the formation of 5-HIAA decreased but simultaneously the formation of 5-HT-O-glucuronide increased (Weissbach et al. 1961a). When ^{14}C 5-HT was administered in situ to tied-off sections of the jejunum of a cat pretreated with nialamide the quantity of absorbed ^{14}C 5-HT in the mucosa increased 35-fold and that found in the venous effluent increased sixfold, while their ^{14}C 5-HIAA content decreased to about 1/5 of that in an untreated cat (Lembeck et al. 1965).

The product of 5-HT oxidative deamination is 5-hydroxyindoleacetaldehyde, which is further oxidized into 5-HIAA (Titus and Udenfriend 1954) or may be reduced to the corresponding alcohol, 5-hydroxytryptophol (Kveder et al. 1962), both of which may be conjugated. Aldehyde can also be converted into polymeric pigments or non indolic oxidation products. Erspamer (1955) was unable to demonstrate the excretion of 5-HIAA in the urine of herbivores either normally or after 5-HT administration; in carnivorous and omnivorous mammals it was the major metabolite. After 5-HT administration in vivo Nakai (1958) found that only a small percentage was recovered in the form of 5-HIAA in rabbit and guinea pig; in vitro liver tissue yielded over 75 % and intestine, lung and brain homogenates under 35 % as 5-HIAA. In these species 5-hydroxyindoleacetaldehyde produced two types of pigment. This reaction was probably catalysed by enzymes of the catalase peroxidase system.

Role of MAO in the inactivation of the catechol amines. MAO inhibitors increased the noradrenaline content of the brain of the rabbit (Shore et al. 1957, Spector et al. 1958) and the rat (Crout et al. 1961); no increase was found in the cat or dog brain (Vogt 1954, Spector et al. 1960). After MAO inhibition, the noradrenaline content of the heart rose in the guinea pig and mouse (Pletscher 1958) and the rat (Crout et al. 1961). Tissue noradrenaline is present in several pools (Trendelenburg 1960, Kopin and Gordon 1962). It can be discharged from the more labile pools

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by tyramine and sympathetic nerve stimulation when it is liberated mainly as free noradrenaline which is O-methylated extracellularly (Kopin and Gordon 1962, Hertting and Axelrod 1961). The most stable noradrenaline pool can be liberated by reserpine and this catechol amine is metabolized intracellularly mainly by deamination. Since only a part of the bound noradrenaline is activated by nerve stimulation, the deaminated products of the bound noradrenaline are predominantly excreted metabolites (Kopin and Gordon 1962, 1963).

Of the circulating adrenaline and noradrenaline administered and probably also endogenously released, approximately 68 % is O methylated and 23 % is deaminated oxidized or reduced and most of this is then O methylated (Kopin 1960). MAO inhibition did not modify the disappearance of administered adrenaline or of noradrenaline (Axelrod and Laroche 1959, Kopin et al. 1961), neither did it prolong their effects (Griesemer et al. 1953).

Brain dopamine is also at least partly metabolized by MAO since the dopamine content of the rabbit brain increased after iproniazid treatment (Carlsson et al. 1958) and in several species 3,4-dihydroxyphenylacetic acid disappeared from the brain (Rosengren 1960b). In the metabolism of administered dopamine in the rat, the main pathway is deamination by MAO (Goldstein and Musacchio 1963).

Other amines. MAO inhibition prolonged the action of administered phenylethylamine and tyramine (Griesemer et al. 1953). MAO inactivated tyramine and tryptamine administered at the effector cell level (Belleau et al. 1961).

MATERIAL AND METHODS

Animals

Guinea pigs were purchased from the farm of Orion Oy Helsinki where they had been bred under standardized conditions for several generations The animals were fed ad libitum with guinea pig pellets (Orion Oy) (containing 3.3 % raw fat 22.1 % raw protein 7.0 % raw fibre 44.8 % nitrogen free extractable substances 9.1 % ash and 13.7 % water) hay and swedes.

The guinea pig mothers were 12 ± 3 month-old females that had been pregnant before At the beginning of the study vaginal smears were examined (Stockard and Papanicolaou 1917) and each female in oestrus was placed in an individual cage with a male for two days The resulting foetuses comprised the main material for measurements of foetal 5-HT contents The age of other foetuses was estimated from their weights according to the present values and Draper's (1920) which were closely similar The equivalence of the ages of the foetuses in different parts of the study can been seen from Table 1 The material of 3 hour-old guinea pigs comprised only animals born under continuous observation The real age of the 1-day-old guinea pigs ranged from 24 to 36 hrs The mean weights of the young guinea pigs in this material were 77 g for 3-hour-olds and 89 g for 1-day-olds At the ages of 1 3 and 9 weeks the values were 130 250 and 500 g respectively The mean weight of adult non pregnant females was 870 g and of males 990 g

Table 1 Weights of guinea pig foetuses in different parts of the study

Mean ± S D M

Material	Weight g at the foetal age of days				
	25	35	45	55	67
5 HT contents	0.58 ± 0.44	3.0 ± 0.9	18 ± 4	42 ± 8	93 ± 15
5 HTP decarboxylase activities	0.92 ± 0.30		24 ± 4		95 ± 17
MAO activities	0.84 ± 0.53		18 ± 7		94 ± 24
5 HTP administration	0.60 ± 0.48				79 ± 18
Value of Draper[1]	0.47	4.2	15	46	74

[1] Calculated from the values given by Draper (1920) Mean weight of guinea pig foetuses on the day indicated ± 1 day Value in the last column refers to 63 ± 1 day-old foetuses

An adult control was examined simultaneously with each series of developing animals In the case of the foetuses the mother served as a control In the full term pregnant guinea pigs aged 12 ± 3 months the 5-HT contents of the brain duodenum and blood and the 5 HTP decarboxylase and MAO activities of the brain duodenum kidney and liver differed only by a very small percentage in both directions if at all from those in the non pregnant female guinea pigs of the same age which had given birth not less than 2 months earlier The only exception was the 5 HTP decarboxylase in the kidney which was 20 8 % lower in the pregnant animals the difference not being significant (Tissari unpublished) For young guinea pigs either their mothers or non pregnant females of similar age were used as controls

The animals were killed daily between 10 00—17 00 hrs

Drugs and Chemicals

The following compounds were used L-adrenaline hydrogen tartrate (C. H Boehringer Sohn Ingelheim am Rhein) synthetic bradykinin (Sandoz A G Basel) 2 bromolysergic acid diethyl amide (BOL 148 Sandoz A G Basel) chymotrypsin cryst (Worthington Biochemical Corporation Freehold N J) disodium edetate (ethylenediaminetetraacetate) (Titriplex[R] III E Merck AG Darmstadt) heparin sodium (Medica Helsinki) 5 hydroxytryptamine creatinine sulphate (F Hoffmann La Roche & Co A G Basel Fluka AG Buchs SG) DL-5-hydroxytryptophan (F Hoffmann La Roche & Co A G Basel Sigma Chemical Company St Louis Miss) iproniazid phosphate (Hoffmann-La Roche & Co A G Basel) D lysergic acid diethylamide tartrate (Delysid[R] Sandoz A G Basel) methysergide (Deseril[R] Sandoz A G Basel) L-noradrenaline hydrogen tartrate (O Star Ab Tampere) pyridoxal 5 phosphate cryst (F Hoffmann La Roche & Co A G Basel Sigma Chemical Company St. Louis Miss) scopolamine hydrobromide (C. H Boehringer Sohn Ingelheim am Rhein) semicarbazide hydrochloride (E Merck AG Darmstadt) and tranylcypromine sulphate (Smith Kline & French Labs Philadelphia)

Other chemicals were commercially available reagent grade products

The doses of 5-HT adrenaline and noradrenaline are given in terms of the base all others in terms of the salt

Estimation of 5-Hydroxytryptamine

Preparation of Tissue Extracts

5-HT was extracted from the tissues by the method of Amin et al (1954) using 95 % acetone This extraction method has recently been recommended by Twarog (1961) for use in connection with the bioassay of 5-HT on rat stomach or uterus However 5 HT recoveries as low and variable have been reported with 95 acetone extraction (Correale 1956 Bogdanski et al 1957 ... mer and Bertaccini 1962) I performed some series of 5 HT recovery tests with 95 acetone from guinea pig brain homogenates In three series the mean and S D M of the recoveries I added 5-HT were 55 ... 5 5 69 6 13 1 and 65 5 9 3 % From a pooled brain homogenate several parallel extractions and estimations of its 5-HT content were also made and in two series of 4 replicates the standard deviations were 2 1 and 4 9 % respectively from the mean 5 HT content found Using different acetone extraction procedures I noted that the 5-HT content of the pooled brain homogenates obtained with 90 acetone was similar to and those with 80 acetone Correale 1956) and with the acetone heptane method (Karki and Paasonen 1959) about 30 greater than that found with 95 acetone From pooled homogenates of guinea pig duodenum 95 90 and 80 acetone extraction gave similar 5-HT contents but the acetone heptane method gave a 5-HT content only 1/3 as high as the others

The guinea pigs were anaesthetized with 1.5 g/kg of urethane given intraperitoneally 5 min before Caesarean section or sacrifice. Foetuses were obtained by Caesarean section and before delivery the throat vessels of the foetuses were severed and the blood collected in siliconized glass tubes containing heparin. The foetuses were decapitated before the umbilical vessels were severed. After delivery the throat vessels of the mother guinea pig were severed and the blood collected in heparinized tubes. To the blood samples 20 vol. acetone was immediately added. The tissues were removed without delay but until this could be done the carcasses were kept at 4 C.

The brains included the medullae oblongatae cerebella and olfactory lobes but not the pituitaries. In the gastrointestinal tract the duodenum was taken as the object of study, preliminary tests having shown it to be the richest 5-HT source there even in young guinea pigs. The duodenum samples comprised a piece 8 cm long taken aborally of the pylorus in the adults, a piece 5 cm long in the 9- to 1 week-old guinea pigs and a piece 3 cm long in the 1-day-olds and all the foetal age groups except the 25-day-old foetuses. The duodenum was opened and blotted dry with filter paper. In the 25-day-old foetuses the whole intestine aborally of the pylorus was taken and treated unopened. The tissues were ground fine in a mortar with 20 ml acetone/g tissue and sand.

The acetone extracts were left to stand for 4 hrs at room temperature being shaken occasionally and then at 4 C ad 24 hrs. The brain and duodenum samples were re-extracted for 1 hr with 20 ml 95 acetone/g tissue, the blood samples were not re-extracted. They were filtered and the filtrates combined. The combined filtrates were evaporated in vacuo at 35 C to a volume of approximately 1 ml. The aqueous residues of the brain and duodenum samples were extracted twice and that of the blood samples once with 10 ml petroleum ether (b.p. 40—60 C). The aqueous residues were evaporated to dryness in vacuo at 35 C. The dry residues were stored at —17 C and assayed during the next two days. When 2 series of such dry residues were stored at —17 C for 10 days 100 % of the original 5-HT content was found in brain and duodenum extracts; in the whole blood extracts the percentages of 5-HT still present were 8[illegible] and 80.

Blood samples for measuring the 5-HT content of platelets were taken from the carotid artery in the adult and 9-week-old guinea pigs and from the abdominal aorta in the younger animals through a polythene catheter straight into polystyrol tubes containing about 1/9 vol. of ice-cold 1.5 disodium edetate in 0.7 % NaCl. A platelet count (Feissly 1951) was made on the whole blood. Platelet rich plasma was obtained by centrifuging the blood at 1500 rev/min (300 g) for 5 min at room temperature. The plasma was withdrawn and the procedure repeated twice. A platelet count was made on the combined plasma (Feissly 1951). The platelet rich plasma was extracted twice with acetone and treated once with petroleum ether. Until extraction only plastic vessels were used. Duplicate assays were made of the platelets of all except the 1-day-old guinea-pigs. The mean deviation of the duplicate assays was 3.1 from the mean of the duplicates and the standard deviation of the mean deviation ± 3.3

Bioassay and Identification of 5-Hydroxytryptamine

The 5-HT in the extract was assayed by Vane's rat stomach method (1957).

170—190 gram rats of both sexes were used without preliminary starvation. The bathing solution was a modification of the rat uterus Ringer of Gaddum et al. (1949): 9.0 g/l NaCl, 0.42 g/l KCl, 0.18 g/l $CaCl_2$, 0.5 g/l $NaHCO_3$, 0.5 g/l glucose, water passed through a column of an anionic and cationic exchange resin and distilled once. The solution contained scopolamine 10⁻ and was gassed with oxygen at 37 C. Load of lever 1.5 g, magnification 1:10. Paton's (1957) pendulum with 1 g weight on lever. Organ bath 10 ml. The preparation was left to stand for

about 1 hr before the beginning of bioassay under slow perfusion with bathing solution through the organ bath 2 standard doses of 5 HT usually of the order of 1—2 ng were employed the 0 9 % NaCl solution of tissue extracts was compared with the standards 4—6 times The responses of the preparation were stable from the start and remained unchanged for about 60 stimulations during which time the base line of the preparation was also constant By using standardized conditions quite similar preparations were obtained each time A typical preparation is illustrated in Fig 1 When necessary stomach preparations could be used even with doses of 0 1 ng 5-HT Recently Uuspaa has described his experiences with the rat stomach preparation in detail (Uuspaa and Uuspaa 1962)

Adrenaline and noradrenaline caused relaxation in the rat stomach preparation In doses about 3 fold the 5 HT dose adrenaline reduced the effect of 5 HT by 50 % and in doses about 10-fold the 5 HT dose inhibited the 5 HT effect totally The respective doses for noradrenaline were about 5 and 15 fold the 5 HT dose Bradykinin induced contraction and the stomach strip was on the average 1/6th as sensitive to it as to 5 HT

Extracts from each series were also tested after the rat stomach preparation had been treated with a 5 HT antagonist The following antagonists were used LSD 10^{-7} BOL 5×10^{-7} and methysergide [illegible] $\times 10^{-8}$

To eliminate any interference by substance P chymotrypsin incubations of tissue extracts were prepared (Pernow 1955) The incubation mixture contained 1 ml 0 9 % NaCl solution of the tissue extract corresponding to roughly 1 g brain or 50—100 mg duodenum tissue 1 ml 0 15 M phosphate buffer pH 7 6 0 6 ml of 50 µg/ml chymotrypsin solution and 0 9 % NaCl ad 3 ml Pre incubation 10 min chymotrypsin incubation 60 min at 37 C The reaction was stopped by placing the incubation bottles in a boiling water bath for 10 min The incubation mixtures were assayed for 5 HT on rat stomach and checked on methysergide treated rat stomach *The control samples contained 500 ng bradykinin instead of tissue extract After the incubation* all the bradykinin was inactivated

The adrenaline and noradrenaline contents of the tissue extracts were checked by polyphenol

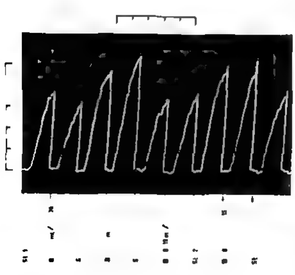

Fig 1 Rat stomach strip used to assay 5 HT Bath volume 10 ml 6 min between each stimulation Drum turned off during washing the bath

[illegible] standard doses of 5 HT (St 1 = 1 3 ng St 2 = 2 ng) were used to which doses of tissue extracts were compared (B = brain extract corresponding to 4 1 g tissue) The preparation was used during a period of 70 stimulations and for heights of contractions caused by St 1 the mean $\pm$ S D M was 35 0 $\pm$ 1 1 mm during the first half of the assay period and 30 5 $\pm$ 2 8 mm during the latter half For heights of St 2 contractions the values were 46 1 $\pm$ 1 9 mm and 42 1 $\pm$ 2 5 mm respectively

Vertical scale in cm on the kymograph Time in min

oxidase treatment (Carven 1956) The mushroom juice was prepared from fresh cultivated mushrooms (*Agaricus (Psalliota) bisporus*) The incubation mixture comprised 0 9 ml 0 9 % NaCl solution of tissue extract corresponding to approx 1 g brain tissue or 50—100 mg duod num and 0 1 ml mushroom juice 1 4 in 0 9 NaCl. The pH of the mixture was about 5 It was incubated at room temperature for 40 min then assayed with rat stomach between 40 and 60 min from the start of incubation The control samples contained 100 ng 5-HT and 200—500 ng adrenaline or 500 ng noradrenaline and during incubation only the catechol amines became inactivated with no reduction in the amount of 5-HT

Measurement of 5-Hydroxytryptophan Decarboxylase Activity

5-HTP decarboxylase was determined by measuring with bioassay the 5 HT formed by ncubating 5 HTP with tissue homogenate directly from the unextracted incubation mixture Since the aim was to examine the occurrence of 5-HTP decarboxylase activity in the guinea pig tissues quantitatively during development an effective and specific MAO inhibitor was required. It was also necessary to ascertain the proportions of enzyme substrate quantities at which activity was highest and on which a comparison between the activities in the tissues of adults and devel oping animals could be based Test conditions similar to those of West (1958) were used

The MAO inhibitor was selected after 5-HTP decarboxylation tests had been made to compare the 5-HT quantities obtained with iproniazid 2×10^{-4} M and 10^{-3} M and pheniprazine 10^{-4} M as MAO inhibitors The tissues studied were the duodenum, kidney and liver of adult guinea pigs and full term foetuses 200 mg of tissue and 400 µg of substrate DL-5-HTP were used The largest quantities of 5-HT were obtained using iproniazid 10^{-3} M iproniazid 2×10^{-4} M was insufficient as an MAO inhibitor in all the samples and pheniprazine 10^{-4} M inhibited 5-HTP decarboxylation in the maternal duodenum and liver and in all the foetal samples When 200 mg kidney homogenate was incubated in the presence of iproniazid 10^{-3} M with a 5-HT amount equimolar to the 5-HTP dose used the disappearance of 5-HT was prevented entirely The 5 HTP decarboxylation activity of the same tissues was then studied using 200 100 and 50 mg tissue with 400 µg DL-5-HTP as substrate and iproniazid 10^{-3} M as the MAO inhibitor 100 mg duodenum and kidney and 200 mg liver were found to consume all the substrate during incubation lasting 30 min. The amount of 5-HT formed below maximum substrate consumption was found to be proportional to the quantity of tissue homogenate used The results of these experiments will be published in detail elsewhere

The procedure used to determine 5-HTP decarboxylase activity in the different age groups was as follows 1—2 g brain tissue — comprising half a brain in adults and young guinea pigs the whole brain of full term foetuses and pooled brains of several younger foetuses — was homogenized in an all glass Potter Elvehjem homogenizer with 2 vol of 0 067 M phosphate buffer pH 8 0 400—1 000 mg kidney tissue — half a kidney from adults a whole kidney from young animals or full term foetuses and pooled kidneys of younger foetuses — was homogenized with 4 vol buffer A piece weighing 50 mg was taken along the whole length of the duodenum from adults and full term foetuses 50 mg of duodena from several 45-day-old foetuses and pooled whole intestines from several 25-day-old foetuses These were finely ground in a mortar with 1 ml buffer and a little sand Similarly a 50 mg piece of liver tissue comprising several pooled livers in the 25-day-old foetuses was also ground

Aliquots of tissue homogenates corresponding to 200 mg brain and 50 mg kidney duodenum and liver were transferred to 25 ml beakers containing 0 1 ml of 1 mg/ml pyridoxal phosphate solution and 0 1 ml of an iproniazid solution corresponding to a final concentration of 10^{-3} M The volumes in the beakers were made up to 4 6 ml with the phosphate buffer with which the

mortars had been washed. The samples were pre-incubated in a metabolic shaker at 37° C i air atmosphere for 20 min, and, after addition of 0.4 ml of 1 mg/ml DL-5-HTP solution, i bated for 30 min. The reaction was stopped by adding N HCl to reduce the pH to 2. The inc tion mixtures were centrifuged immediately and the supernatants removed and stored at —1 The 5-HT content of the samples was assayed directly without extraction on rat stom within 1—2 days, during which time the 5-HT in the samples had been found not to decr The samples were thawed, diluted and adjusted to about pH 7 immediately before being ad to the organ bath and a fresh dilution was made for each stimulation. The incubation solut caused no contractions in rat stomach treated with 5-HT antagonist. Control samples prep without addition of enzyme produced no contraction of the stomach preparation in doses responding to 20 μg DL-5-HTP, neither did incubation mixtures prepared with boiled enz 5-HTP decarboxylase activity is expressed as μg of 5-HT formed/g of tissue per 30 min.

Measurement of Monoamine Oxidase Activity

MAO was assayed by the method of Sjoerdsma et al. (1955) in which 5-HT reduction du the incubation of 5-HT with a tissue homogenate is measured. 5-HT was determined by assay from the unextracted incubation mixture.

Here, too, the object was a quantitative analysis of MAO activities in the tissues of develo animals. To ensure the most reliable comparison between the tissues of adult and develo guinea pigs, it was necessary to find the test conditions under which enzyme activity was grea The MAO activities of the kidney, liver and duodenum were studied in adult guinea-pigs full-term foetuses under the experimental conditions of Bogdanski et al. (1957) using 200 100 mg and 50 mg of tissue, with 4 μmol 5-HT as substrate. In all the tissues, both adult foetal, the greatest MAO activity per gram of tissue and per 30 min was obtained with 50 of tissue, in which case the consumption of substrate was of the order of 60 % in the adult tis and foetal duodenum and —% in the other foetal tissues. The greater the quantity of ti employed and thus the higher the percentage of substrate consumed, the lower was the M activity found per gram of tissue. In the light of these results, which will be published discussed elsewhere, 50 mg was taken as the quantity of kidney, liver and duodenum tissue 4 μmol 5-HT as the amount of substrate for the measurement of MAO activity in the diffe age groups.

The brain, kidney, liver and duodenum homogenates were prepared in the same manne for the determination of 5-HTP decarboxylase activity, except that the tissues were homogen in water. Amounts of homogenates corresponding to 200 mg brain and 50 mg kidney, duoden and liver were placed in beakers containing 0.3 ml of 0.5 M phosphate buffer, pH 7.4. The v umes were made up to 2 ml with water with which the mortars had been washed. The samp were pre-incubated in a metabolic shaker at 37° C in an air atmosphere for 20 min, and 1 m 4 μmol/ml 5-HT solution was added. They were then incubated for 30 min. The reaction v stopped by adding N HCl to reduce the pH to 2. The incubation mixtures were centrifuged im diately and the supernatants removed and stored at —17° C. The 5-HT remaining in the sam was assayed directly without extraction on rat stomach, within 1—2 days, during which t no loss had been found in their 5-HT content. The samples were thawed, diluted and adjus to about pH 7 immediately before being added to the organ bath, and a fresh dilution was m for each stimulation. The samples caused no contractions in rat stomach treated with 5- antagonist. For each sample a control sample was prepared in which the reaction was stop immediately after addition of the 5-HT. The 5-HT recovery in these samples was 100 % .

the 5-HT disappearance during incubation was calculated against these control values MAO activity is expressed as µg 5-HT metabolized/g of tissue per 30 min

Specificity Tests

To test the specificity of the MAO determinations samples of adult and foetal brain duodenum kidney and liver tissues were prepared to which tranylcypromine 10^{-4} M or semicarbazide 10^{-3} M or 10^{-4} M (final concentrations) was added before pre incubation Samples to which no inhibitor was added served as controls After the incubation period a 1 ml aliquot of each sample was removed and the 5 HT extracted and determined fluorimetrically by the method of Bogdanski et al (1956) Control samples were also prepared to which 5 HT was only added at the end of incubation and from these samples the quantity of 5 HT destroyed during incubation was calculated

5-Hydroxytryptophan Administration Experiments

The guinea pig mothers were anaesthized with urethane 1—1 2 g/kg intramuscularly or intraperitoneally During the tests a warmed operation stand was used

For the experiments in which the effect of maternal 5 HTP administration on the foetal 5 HT contents of the brain duodenum and whole blood or whole body was studied a polythene catheter was placed in the jugular vein of the mother through it the 5 HTP (20 mg DL 5-HTP/kg dissolved in 0 9 % NaCl) was administered and maternal blood samples were collected in heparinized tubes The control foetus was removed by Caesarean section before 5 HTP administration and the others removed after 5 HTP administration once or several times The foetal blood samples were collected from the severed throat vessels The foetal and maternal tissues were extracted and assayed with rat stomach for 5 HT as described above

In later experiments in which the 5 HT contents of the platelets and plasma were assayed separately the 5 HTP was administered and the maternal blood samples taken through a polythene catheter in the carotid artery Blood samples were collected straight into polystyrol tubes containing 1/9 vol of ice-cold 1 5 % disodium edetate in 0 7 % NaCl After the uterus was opened the foetuses were placed on the abdominal wall of the mother Where the umbilical circulation was intact a blood sample was drawn from the abdominal aorta of the foetuses into a nylon syringe containing approx 1/9 vol anticoagulant 3—5 ml blood was thus obtained from full term foetuses With this technique of blood collection it was only possible to open the uterus once The blood samples were stored at 4 C and centrifuged at 4 C and 1200 rev/min (170—200 g) for 20 min to prepare platelet rich plasma The platelets in the latter were counted (Feissly 1961) and the platelets and plasma separated by centrifugation at 4 C and 3000 rev/min (1400 g) for 30 min The platelet free plasma was removed and checked microscopically The platelet pellets were mixed and homogenized with 0 5 ml water and transferred to beakers with tube washing water ad 3 ml The platelet homogenates and platelet free plasma were extracted with 20 vol acetone re-extracted with 20 vol 95 % acetone and the aqueous residue treated once with petroleum ether The extracts were assayed on rat stomach and the contracting activity checked with 5 HT antagonist

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All values in the study are expressed on a wet weight basis

mortars had been washed The samples were pre incubated in a metabolic shaker at 37 C in an air atmosphere for 20 min and after addition of 0 4 ml of 1 mg/ml DL-5 HTP solution incubated for 30 min The reaction was stopped by adding N HCl to reduce the pH to 2 The incubation mixtures were centrifuged immediately and the supernatants removed and stored at —17 C. The 5-HT content of the samples was assayed directly without extraction on rat stomach within 1—2 days during which time the 5-HT in the samples had been found not to decrease The samples were thawed diluted and adjusted to about pH 7 immediately before being added to the organ bath and a fresh dilution was made for each stimulation The incubation solutions caused no contractions in rat stomach treated with 5-HT antagonist. Control samples prepared without addition of enzyme produced no contraction of the stomach preparation in doses corresponding to 20 µg DL-5-HTP neither did incubation mixtures prepared with boiled enzyme 5-HTP decarboxylase activity is expressed as µg of 5-HT formed/g of tissue per 30 min.

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Here too the object was a quantitative analysis of MAO activities in the tissues of developing animals To ensure the most reliable comparison between the tissues of adult and developing guinea pigs it was necessary to find the test conditions under which enzyme activity was greatest. The MAO activities of the kidney liver and duodenum were studied in adult guinea pigs and full term foetuses under the experimental conditions of Bogdanski et al (1957) using 200 mg 100 mg and 50 mg of tissue with 4 µmol 5-HT as substrate In all the tissues both adult and foetal the greatest MAO activity per gram of tissue and per 30 min was obtained with 50 mg of tissue in which case the consumption of substrate was of the order of 60 % in the adult tissues and foetal duodenum and 40 in the other foetal tissues The greater the quantity of tissue employed and thus the higher the percentage of substrate consumed, the lower was the MAO activity found per gram of tissue In the light of these results which will be published and discussed elsewhere 50 mg was taken as the quantity of kidney liver and duodenum tissue and 4 µmol 5-HT as the amount of substrate for the measurement of MAO activity in the different age groups

The brain kidney liver and duodenum homogenates were prepared in the same manner as for the determination of 5 HTP decarboxylase activity except that the tissues were homogenized in water Aliquots of homogenates corresponding to 200 mg brain and 50 mg kidney duodenum and liver were placed in beakers containing 0 3 ml of 0 5 M phosphate buffer pH 7 4 The volumes were made up to 2 ml with water with which the mortars had been washed The samples were pre incubated in a metabolic shaker at 37 C in an air atmosphere for 20 min and 1 ml of 4 µmol ml 5-HT solution was added. They were then incubated for 30 min The reaction was stopped by adding N HCl to reduce the pH to 2. The incubation mixtures were centrifuged immediately and the supernatants removed and stored at —17 C. The 5 HT remaining in the samples was assayed directly without extraction on rat stomach within 1—2 days during which time no loss had been found in their 5-HT content The samples were thawed diluted and adjusted to about pH 7 immediately before being added to the organ bath and a fresh dilution was made for each stimulation The samples caused no contractions in rat stomach treated with 5-HT antagonist. For each sample a control sample was prepared in which the reaction was stopped immediately after addition of the 5-HT The 5-HT recovery in these samples was 100 and

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All values in the study are expressed on a wet weight basis

Statistical Methods

The arithmetic means and S E M or S D M were calculated The significance of the differences between the groups of developing animals and adults or between different developing groups was determined by Student s t test on the assumption that the variations of the different age groups are basically equal

5-HYDROXYTRYPTAMINE, 5-HYDROXYTRYPTOPHAN DECARBOXYLASE AND MONOAMINE OXIDASE IN GUINEA-PIG TISSUES DURING FOETAL DEVELOPMENT[1]

5-Hydroxytryptamine

The 5-HT contents of the brain and duodenum were examined at intervals of ten days in foetuses between the 25th day and full term (67th day). The only blood samples analysed were those from the 55- and 67-day-old foetuses. For the brain and the intestine the samples studied on the 25th day were pooled from all the foetuses in the litter, the intestine samples comprising the whole intestine aborally of the pylorus. In the other age groups every foetus of the litter was studied separately. The results are shown in Table 2.

Even in the 25-day-old foetuses the average 5-HT content of the brain was 16 ng/g, which was 10.9 % of the adult value. The content rose slightly up to the 45th foetal day. Between then and the 55th day it doubled, and nearly doubled again by the 67th day, bringing the value up to 50.5 % of the adult level. Both the latter increases are statistically significant ($P < 0.01$). The brain 5-HT content of the full term foetuses was still significantly different from that of the adults ($P < 0.001$).

The 5-HT content found in the intestine on the 25th foetal day was roughly the same as the content in the brain at that stage; it was only 0.11 % of the content in the adult duodenum. The average content on the 35th day was 20 times as great, but was still only 2.1 % of the adult content. Despite the correctness of the foetal age, the individual values varied greatly at that time. Between the 35th and 45th days the increase was almost 20-fold ($P < 0.001$). By the 45th day the level was 37.2 % of the adult level. By the 55th day the increase had slowed down — relative content 45.5 % — and this was the last time that the difference from the adult level was significant ($P < 0.001$). By the 67th foetal day the content had almost doubled again ($P < 0.001$) and reached 86.1 % of the adult level.

The relative brain and duodenum 5-HT levels shown in the table have been calculated with respect to the average values for all the mothers of the foetuses examined. When each age group of foetuses was compared with the mothers of the group studied simultaneously, roughly the same percentages were found in

[1] A preliminary report on 5-HT contents of guinea-pig tissues during foetal development was made in 1960 (Tissari 1960) and on 5-HTP decarboxylase and MAO activities in 1963 (Tissari 1963). The results presented in this section have also been communicated in two other papers (Tissari 1964, 1965).

Table 2 5 HT contents of guinea pig tissues during foetal development

Values of foetuses in the same litter are given on the same line

Foetal age	Brain: 5 HT content ng/g	Brain: % of adults	Duodenum: 5 HT content ng/g	Duodenum: % of adults	Blood: 5-HT content ng/ml	Blood: % of adults
25 days	20 0		20 0			
	23 3		12 2			
	16 7		4 8			
	14 1					
	15 9		14 5			
	8 2					
	16 6		14 3			
	12 8		11 0			
Mean ± S E M	[a]16 0 ± 1 6	10 9	[a]12 1 ± 2 3	0 11		
35 days	16 4 8 3		152 15			
	16 6 21 8 20 8		60 66			
	30 1 36 3		938 455			
	12 2					
	21 0		23			
Mean ± S E M	[a]20 4 ± 2 9	13 9	[a]244 ± 129	2 1		
45 days	26 9 18 6 25 6		6 600 5 280 5 210			
	23 8 16 6		2 960 3 810			
	27 2 23 8 28 8		3 990 2 910 3 550			
Mean ± S E M	[a]23 9 ± 1 5	16 3	[a III]4 290 ± 457	37 2		

age	5 HT content ng/g	% of adults	5 HT content ng/g	% of adults	5 HT content ng/ml	% of adults
55 days					4 0 1 2 1 8 3 8 1 4	
	25 8 37 3 29 4 26 5 26 1		4 170 4 760 3 800 1 670 2 500		4 4 6 0 2 0 2 6 3 1	
	82 8 69 6		8 720 8 100		15 3 20	
	49 4 40 3 54 3		7 320 5 680 6 060		13 4 6 7 8 8	
Mean ± S E M	[a][II]44 2 ± 6 3	30 2	[a]5 280 ± 739	45 5	[a]6 3 ± 1 5	20 8
67 days	34 3 42 9		3 930 3 050		11 5 10	
	78 3 74 2		9 600 10 700		2 4 7 5	
	69 9 38 6		13 600 11 300			
	79 4 81 9 103		9 030 8 000 4 030		7 9 3 6 6)	
					6 2 7 2 7 6	
					5 5 8 7 14 2 8 5	
	72 0		5 410		12 9	
	71 3 55 4		7 280 5 740		4 3 4 3	
	85 5		9 090		) 0	
	92 6 116		13 300 16 700		9 6 13 1	
	102		9 880		14 8	
	74 3 62 5		20 900 17 400		15 7 15 3	
Mean ± S E M	[II]74 1 ± 5 2	50 5	[III]9 920 ± 1 170	86 1	[a]9 0 ± 0 8	29 6
Adults						
[c]N	32		33		29	
Mean ± S E M	147 ± 10 8		11 600 ± 850		31 1 ± 4 2	

The significance of the differences as compared with the adults is denoted by [a] P 0 001

The significance of the differences as compared with the preceding age group is denoted by [II] = P < 0 01 [III] P < 0 001

[c] Number of measurements

Table 3 5 HT content in whole guinea pig foetuses

Foetal age	5-HT content ng/g without treatment	5 HT content ng/g after polyphenol oxidase treatment
25 days	2 5 2 9 3 0 2 6 2 1 2 6	12 7 8 3
Mean ± S E M	2 6 ± 0 13	10 5
35 days	Not measurable — » — — » —	0 7 1 3 10 1
Mean ± S E M		4 0 ± 3 0
45 days	12 6 16 8	 16 8
Mean	14 7	16 8

the brain the 5 HT content in the brain of the 67 day old foetuses was 54 8 % of the mean for their mothers In the duodenum the relative values calculated according to both bases of comparison differed by as much as c 15 % in only two age groups The duodenum 5 HT content of the full term foetuses was 72 3 % of that of their mothers

The blood 5 HT contents of both the foetal groups examined were very low 20 8 % of that of all the mothers on the 55th day and 29 6 % on the 67th day, and 24 3 % and 34 7 % of that of their own mothers

Extracts were made from whole foetuses in the younger groups and the results are shown in Table 3 The extracts of the 25 day old whole foetuses strongly relaxed the rat stomach preparation but a slight contracting activity due to 5 HT was found Some of the extracts were treated with polyphenol oxidase after which they did not relax the rat stomach preparation and an average 5 HT content of 10 ng/g was obtained which approximately resembled the 5 HT contents of the brain and intestine found in the foetuses of the same age Untreated extracts of 35-day old foetuses caused nothing more than a strong relaxation in the rat stomach preparation After polyphenol oxidase treatment the relaxing effect diminished and slight contraction appeared A contracting activity corresponding to about 15 ng/g of 5 HT was discovered in two extracts from 45 day old whole foetuses and this did not increase after polyphenol oxidase treatment

Specificity Tests on Tissue Extracts

In the doses used for assaying 5-HT the contractions produced by the brain duodenum and blood extracts of all the age groups were inhibited entirely when they were tested in a rat stomach preparation treated with a 5 HT antagonist

Table 4 5 HT equivalents of acetone extracts of guinea pig tissues without and after treatment with polyphenol oxidase or chymotrypsin

Tissue and age	Without treatment 5 HT ng/g	After polyphenol oxidase treatment		After chymotrypsin treatment	
		5 HT ng/g	Ratio	5 HT ng/g	Ratio
Brain					
25 day-old foetuses	[1]25 5			[1]18 8	0 74
	25 9	29 6	1 14	47 7	1 83
	18 7	20 3	1 09	37 8	2,05
45-day-old foetuses	[1]37 7	37 7	1 00	[1]39 2	1 04
	[1]25.3	25 7	1 02	[1]31 8	1 26
67 day-old foetuses	71 3	68 8	0 96	[1]38 4	1.38
Adults	[1]172	172	1 00	[1]172	1 00
	[1]192	192	1 00	[1]256	1 33
Mean			1 03		1 33
Duodenum					
25 day old foetuses	22 1			27 7	1 25
	7 0	41 0	5 90	186	26 6
	23 1	20 7	0 90	49 5	2.14
45-day-old foetuses	[1]2 640	2 220	0 84	[1]2 520	0 95
	[1]2 610	2 460	0 95	[1]3 130	1 20
67-day-old foetuses	[1]7 880	8 080	1 03	[1]7 880	1 00
Adults	[1]15 800	13 200	0 84	[1]15 500	0 98
	[1]12 900	12 000	0 93	[1]12 900	1 00
Mean			1 63		4.39

[1] Activity of extract was abolished by methysergide
[2] Activity of extract was not abolished by methysergide

Only in the whole 45 day old foetus extracts did any noticeable activity continue In addition to this lots of pooled extracts of brain and of duodenum were prepared from the tissues of foetuses and adults After being dissolved in 0 9 % NaCl the extracts were divided into three equal parts the first being incubated with polyphenol oxidase the second with chymotrypsin and the third serving as a control without treatment The results of these incubations are given in Table 4 After polyphenol oxidase treatment the 5 HT content of the brain extracts in all the age groups was found to be the same as that of the controls Chymotrypsin treatment did not diminish the rat stomach contracting activity of the brain

samples contrary to expectation, the activity increased somewhat in a large number of samples from all age groups. In the doses used for 5-HT assay (equal to 10–100 mg of tissue) some activity was still found in nearly all the chymotrypsin-treated brain samples when they were tested with rat stomach treated with methysergide.

Neither did the rat-stomach contracting activity of the duodenum extracts increase after polyphenol oxidase treatment except in one 25-day-old foetus. After chymotrypsin treatment the activity was found to have increased in the extracts of intestines of 25-day-old foetuses: these extracts did not suffice for methysergide controls. In the other age groups, unaltered activity was observed after chymotrypsin treatment, and was antagonized completely by methysergide when doses of extracts corresponding to 2 ng of 5-HT were used. However, these duodenum chymotrypsin incubations also caused methysergide-resistant contractions in larger doses equivalent to 10–100 mg of tissue (which corresponded to 2 ng of 5-HT in the 25-day-old foetuses), though untreated extracts of the same amount of tissue did not cause any contraction.

From the results presented, it can be concluded that the 5-HT activity found by the methods of extraction and analysis used here is due entirely to 5-HT and contains no catechol amine or polypeptide components. Any effect of acetylcholine was eliminated by the presence of scopolamine. A small [illegible] of tissue extracts from different age groups was assayed by the oestrous rat uterus method (Amin et al., 1954), and the absolute and relative va[illegible] were of the same order as those obtained using rat stomach. Th[illegible] are not included in Table 2.

Sex Differences in Tissue 5-Hydroxytryptamine

In the 67-day-old foetuses, which [illegible] material, 5-HT contents were estimated for males and [illegible] ± S.E.M. of brain 5-HT contents in 9 female and 9 male [illegible] and 7+5 ± 6. ng/g, respectively, and those of [illegible] 751 ng/g 9120 ± 1,570 ng/g, respectively. T[illegible] and S.E.M. were 83 ± 13 ng/ml in 12 female [illegible] ± 11 foetuses. The differences between the [illegible] were not significant. In a small [illegible] tions that the tissue 5-HT contents [illegible] of the latter.

[illegible]

The 5-HTP decarboxylase activity [illegible] were examined on the liver, [illegible] studied simultaneously [illegible] one [illegible] examined. The results are shown in T[illegible]

The 5-HTP decarboxylase activity [illegible] 2×10^{-4} M, as well as in [illegible] and [illegible] old foetuses, only 2×10^{-4} [illegible] using the stronger inhibitor was 3 [illegible] between the foetal and adult values

Table 5 5 HTP decarboxylase activities of guinea pig tissues during foetal development
μg 5-HT formed g of tissue/30 min

Foetal age	Brain 5-HTPD activity[4]	Brain % of adults	Brain 5-HTPD activity[5]	Brain % of adults	Duodenum 5-HTPD activity	Duodenum % of adults	Kidney 5-HTPD activity	Kidney % of adults	Liver 5-HTPD activity	Liver % of adults
25 days	3 15				15 00		9.28		364	
	2.50				6 26				533	
	2.50				11.30		3.32		440	
	2.50				5 00		4 63		273	
									348	
									230	
Mean ± S E. M	[2]2.66 ± 0 16	26 4			[2]9 39 ± 2.31	1 1	[2]5 74 ± 1 81	0,22	[2]365 ± 45	36 8
45 days	2,42		3 50		667		100		210	
	1 48		2.32		684		130		182	
	2.50		3 13		637		66 7		217	
	2.50		3.24		978		147		200	
Mean ± S E. M	[2]2,23 ± 0 24	22.0	[2]3 05 ± 0 25	22.4	[III]742 ± 79	83 6	[2,II]111 ± 18	4.3	[2]202 ± 8	20 4
67 days	6 67						1 400		389	
	4.33				1,500		1 430		867	
	6 82				1,250		2,400		750	
	6 50		8.34		1 000		1 030		733	
	7 00		9 00		1 050		1,530		952	
	6 67		11 00		520					
	7 15		11 80							
	5 12		7 03							
	5 00		6 00							
Mean ± S E. M.	[2,III]6 15 ± 0 34	67 9	[III]8 86 ± 0 91	65 [illegible]	1 060 ± 162	121	[2,III]1,560 ± 227	60 4	[2,III]738 ± 96	74 5
Adults (N)[6]	17		9		13		[illegible]		15	
Mean ± S E. M.	10 1 ± 0.39		13 6 ± 1 0		887 ± 73		2,580 ± 174		990 ± 56	

The significance of the differences as compared with the adults is denoted by [1] = $P < 0.05$ [2] = $P < 0.001$

The significance of the differences as compared with the *preceding age group* is denoted by [II] = $P < 0.01$ [III] = $P < 0.001$

[4] Iproniazid 2×10^{-4} M as MAO inhibitor

[5] Iproniazid 10^{-4} M as MAO inhibitor

[6] Number of measurements.

finding confirms the observation, presented below that brain MAO activity was almost the same in the adult and foetal groups The brain 5 HTP decarboxylase activity in the 25 and 45 day old foetuses was approximately 25 % of that of the adults By the 67th foetal day it had almost trebled to become 65 % of the adult activity This difference was still significant ($P < 0.001$)

The 5 HTP decarboxylase activity in the 25 day old foetus duodenum and kidney was very small 1 1 and 0 22 % of the adult levels This was the only time the 5 HTP decarboxylase activity in the foetal duodenum differed significantly from that of the adults ($P < 0.001$) After this it increased very rapidly up to the 45th foetal day — almost 100 fold attaining 83 6 % of the adult activity By full term it had increased by c 40 % to attain 121 % of the adult mean

The 5 HTP decarboxylase activity of the kidney developed more slowly By the 45th foetal day it had risen from a barely detectable value to 111 μg/g/30 min corresponding to 4 3 % of the adult level Between then and the 67th day it rose much higher, increasing 15 fold to 60 4 % of the adult level The difference between the full term foetuses and adults was significant ($P < 0.02$)

The activity found in the liver of the 25 day old foetuses was of quite a different order from the above tissues It averaged 365 μg/g/30 min — 36 8 % of the adult controls In the 45 day old foetuses it had fallen to almost half this level Between the 45th and 67th day it rose again nearly 4 fold and reached 74 5 % of the adult level — still a significant difference ($P < 0.05$)

The adult control used for calculating the relative activities of the foetal tissues was the *mean* activity of all the mothers of the foetuses In each foetal age group the foetuses and adults were of the same number, so it was possible to compare the foetus values with those of the mothers of the same age group Here too the relative values were very similar The brain activity was 26 % of the activity of the simultaneous controls on the 45th foetal day and 58 5 % on the 67th day The percentages for the kidney and liver were almost identical which ever basis of comparison was used The liver activity of the 25 day old foetuses was 34 6 % of the level found in their mothers The decarboxylase activity of the duodenum varied somewhat in different groups of mothers thus the activity of the 45 day old foetuses compared with that of their own mothers was 108 % and that of the 67 day old foetuses 92 2 %

Also studied was the total foetus 5 HTP decarboxylase activity on the 25th day iproniazid 2×10^{-4} M being used as the MAO inhibitor The mean and S E M of 5 HTP decarboxylase activity in 4 samples were 10 ± 2.0 μg/g/30 min The mean activity and S E M of adult brains similarly studied were 9.0 ± 1.0 μg/g/30 min The liver tissue of 25 day old foetuses weighs 5—10 mg per foetus so the 5 HTP decarboxylase activity of the total foetuses depends almost entirely on the activity in this tissue

Monoamine Oxidase

MAO activity like that of 5 HTP decarboxylase was studied in the brain duodenum kidney and liver of 25 45 and 67 day old guinea pig foetuses One foetus and the mother of each litter were examined and all the tissues mentioned were studied simultaneously (Table 6)

Table 6 MAO activities of guinea pig tissues during foetal development

μg 5-HT metabolized/g of tissue/30 min

Foetal age	Brain MAO activity	Brain % of adults	Duodenum MAO activity	Duodenum % of adults	Kidney MAO activity	Kidney % of adults	Liver MAO activity	Liver % of adults
25 days	746		0		3 200		0	
	1,350		0				2,980	
	905		3 040		0		0	
	905		3,250				1 970	
	0		0		0		0	
	588		4 670					
Mean ± S. E. M	749 ± 182	65 9	[3]1 830 ± 229	22.4	[3]1 070 ± 1 070	7 7	[3]990 ± 627	9 9
45 days	390		5 550		605		3,280	
	760		4 690		704		4 690	
	979		676		704		3 970	
	820		7 040		0		4 690	
	468		5 110		1,280		5 550	
Mean ± S. E. M	[2]683 ± 111	60 1	[3]4 610 ± 1 060	56 4	[3]659 ± 203	7.3	[3][III]4 440 ± 382	44 5
67 days	1 760		9 330		4 670		5 630	
	1 010		7 830		3 650		3 040	
	352		3 640		6 250		8 210	
	236		9 810		7,310		4 670	
	352		10 950		6 450		7 830	
	940							
	1 170							
	859							
Mean ± S. E. M.	835 ± 181	73 4	8,310 ± 1 270	102	[III]5 670 ± 660	62 6	[3]5 880 ± 970	59 0
Adults								
[4]N	15		16		17		17	
Mean ± S E M	1 140 ± [illegible]		8 170 ± 551		9 050 ± 297		9 960 ± 451	

The significance of the differences as compared with the adults is denoted by [1] = [illegible] < 0 05 [2] = P < 0 01 [3] = P < 0 001

The significance of the differences as compared with the preceding age group is denoted by [III] = P < 0 001

[4] Numbers of measurements.

On the 25th day similar activity was found in all the tissues an average of 1 000 μg/g/30 min. No differences between the tissues were detected at this stage.

In all the foetal age groups brain MAO activity was characterized by wide variation: differences of twofold or more were found between individuals of the same age group. On the other hand, little deviation was found between the adults. The mean activity in all the foetal age groups was similar, roughly 2/3 of that in the adults. Only the 45-day-old foetuses differed significantly from the adult controls.

In half the 25-day-old foetuses no MAO activity was discovered in the duodenum, kidney or liver; in the other half, there was moderate activity. The 5-HT assay method used was so sensitive that it brought out substrate reductions of less than 10 %. The mean duodenum activity on the 25th day was roughly 20 % of the adult level, after which there was a steady rise. On the 45th day it was 56.4 % of the adult activity ($P < 0.02$). On the 67th day the activity was up to the adult level.

Only 20 mg of tissue was available for studying the kidney MAO activity on the 25th day. To make up this amount, the kidneys of 10—15 foetuses were required. The activity thus revealed was compared with that of the adults obtained using 20 mg of tissue. The mean foetal activity thus detected was 7.7 % of the adult. On the 45th day MAO activity was found in the kidney of nearly all the foetuses examined, the mean being 7.3 % of the adult activity. Only on the 67th day was any rapid increase revealed in the kidney MAO activity: this was about 60 % of the adult level ($P < 0.001$).

Development in liver MAO activity was fastest between the 25th and 45th foetal day, the increase being from 9.9 % to 44.5 % of the adult. After this there was a slower rise up to full term, when the level was c. 60 % of the adult ($P < 0.001$).

In Table 6, too, the relative values of foetal MAO activity are presented in comparison with the mean values for the mothers of all the foetal age group. Using each age group's own mothers, examined simultaneously, as the basis of comparison, the development of relative MAO activities was found to be similar and the percentages themselves almost identical. In the full term foetuses the brain activity was 60 %, and the duodenum activity 95 % of those of their own mother.

The MAO activity of whole 25-day-old foetuses was also determined. The mean and S. E. M. in a series of eight foetuses were 514 ± 168 μg/g/30 min. The mean was roughly 50 % of the activity in the adult brains studied simultaneously.

Throughout the study 50 mg of duodenum, kidney and liver tissue were used in all age groups for determining MAO activity. These quantities of adult tissue metabolized about 60 % of the substrate. In earlier experiments on techniques of MAO determination, using these tissues, I had found that the greater the excess of substrate the higher was the MAO activity found per gram of tissue and per time unit (Tuomisto, unpublished). The relative values for MAO activity so obtained are somewhat higher than the real ones in the foetal age groups in which the activity — and thus the substrate consumption — were low.

Table 7 Specificity test of MAO determination Inhibition of MAO activities of guinea pig tissues in vitro by different drugs

Results expressed as mean values ± S E M Figures in parentheses represent number of measurements

Tissue and age	MAO activity μg 5-HT metab /g of tissue 30 min	Inhibition of MAO activity By tranylcypromin 10^{-4} M	By semicarbazide 10^{-3} M	10^{-4} M
Brain				
25-day old foetuses	508 838 (2)	130 96 8 (2)	32 3 (1)	—39 0 32 3 (2)
45 day-old foetuses	658 ± 81 8 (3)	89 0 11 6 (3)	5 5 16 0 (3)	5 5 16 0 (3)
67 day-old foetuses	1 090 596 (2)	89 4 125 (2)	22 3 —10 2 (2)	22 3 12 3 (2)
Adults	790 ± 78 0 (3)	106 2 6 (3)	10 5 5 0 (3)	8 5 106 (2)
Duodenum				
25 day-old foetuses	184 1 000 (2)	162 176 (2)	27 0 (1)	
45 day old foetuses	3 590 72 3 (3)	156 ± 12 2 (3)	27 0 36 9 (3)	—26 9 (1)
67-day old foetuses	5 600 5 330 (2)	114 136 (2)	—3 8 5 5 (2)	—13 1 5 5 (2)
Adults	6 970 ± 284 (3)	115 3 8 (3)	20 4 ± 3 8 (3)	14 6 11 2 (2)
Kidney				
45 day old foetuses	1 520 ± 227 (3)	75 5 30 8 (3)	30 7 ± 9 4 (3)	3 1 ± 12 1 (3)
67 day old foetuses	5 110 3 720 (2)	90 9 72 6 (2)	—9 6 15 1 (2)	—19 1 7 9 (2)
Adults	7 860 ± 150 (3)	93 0 ± 4 1 (3)	34 1 ± 26 5 (3)	7 0 2 8 (2)
Liver				
25 day-old foetuses	3 220 ± 470 (3)	88 9 ± 12 0 (3)	0 —4 9 (2)	19 5 4 9 (2)
45 day-old foetuses	4 780 ± 216 (3)	76 4 ± 15 0 (3)	15 6 ± 4 1 (3)	—5 8 11 4 (2)
67 day old foetuses	5 810 6 670 (2)	79 4 92 8 (2)	3 7 12 5 (2)	0 12 5 (2)
Adults	9 100 ± 384 (3)	93 0 ± 3 6 (3)	11 8 ± 3 3 (3)	5 8 5 4 (2)

Specificity Tests of Monoamine Oxidase Determination

Table 7 shows the brain duodenum kidney and liver MAO activity in foetuses of different age groups and adult guinea pigs when the incubation mixture contained either tranylcypromine 10^{-4} M or semicarbazide 10^{-3} M or semicarbazide 10^{-4} M There was no reduction of 5 HT during the incubation of the brain and duodenum samples containing tranylcypromine 10^{-4} M in any age group In the kidney and liver samples containing tranylcypromine the inhibition of 5 HT reduction averaged slightly less than 100 % In all the incubation mixtures containing semicarbazide the amount of 5 HT remaining was slightly greater or smaller than that in the control samples This is evidently due to the variations in the 5 HT extraction and to possible interference of the semicarbazide in the fluorimetric determination of 5 HT From the inhibition produced by the tranylcypromine and the lack of effect with the semicarbazide it can be concluded

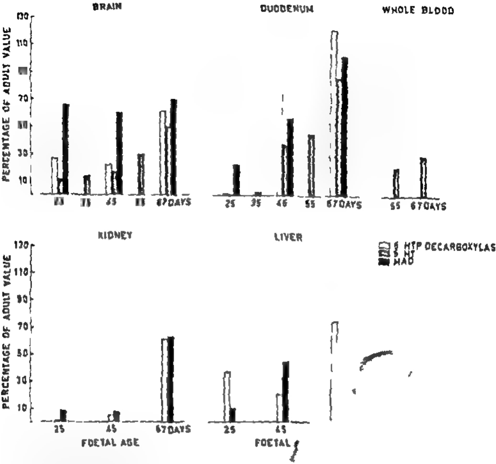

Fig 2 5 HT contents and 5 HTP decarboxylase during foetal development expressed as ... tissues Values on horizontal lines refer to foeta... Tables 2 5 and 6

pig tissues ... in adult ... *from*

that the 5 HT metabolization observed in homogenates of the different age groups

These MAO activity values d... determination did not differ on the who... were obtained by bio-assay of 5 HT C... imetrically measured MAO activity biologically

A summary of the changes in 5 HT cc... MAO activities in guinea pig tissues

Discussion

All species of animals go through a period in which the morphology function and chemical metabolism of the brain develop very rapidly Flexner (1950) calls this the critical period and the age at which it occurs varies in different species and also in different structures of the brain within the same species In the development of the guinea pig cerebral cortex this period occurs between the 41st and 45th foetal day when the neuroblasts of the cortex change into immature neurones

Peters and Flexner (1950) have studied the morphological development of the guinea pig cortex between the 30th foetal day and birth The density of the cell population in the cortex decreases sharply from the 30th to the 44th foetal day being 20 times greater on the former than on the latter On the 44th foetal day it is still 50 % greater than in the full term foetus and in the latter it is twice as great as in the adult The nucleus attains its final volume on the 43rd foetal day The volume of the cell body increases very rapidly up to the 43rd foetal day Then it grows at a slower rate up to full term at which time it is only 15 % smaller than in the adults Nissl s substance appears on the 41st foetal day and increases rapidly up to the 45th foetal day reaching almost the adult level On the 41st foetal day the cortex of the guinea pig foetus can be divided into five layers like that of the adult Between the 41st and 45th foetal days the processes of the nerve cells develop rapidly their relative volume doubles and adult form cells appear in the lower layers of the cortex The number and size of the cell processes continue their rapid increase up to the 53rd foetal day and then up to full term. By this time the cells resemble adult cells

Continuous electrical activity has been registered in the cortex of the guinea pig starting from the 45th foetal day The shape of the electrocorticogram matures up to the end of the foetal period (Jasper et al 1937 Flexner et al 1950) Using an intrauterine technique Bergstrom et al (1962) demonstrated occasional spontaneous electrical activity as early as the 40th foetal day Electrical responses caused by strychnine are fully developed in the guinea pig by the 46th foetal day (Flexner et al 1950) The electrocortical activity of the guinea pig at birth is almost indistinguishable from that of the adult (Jasper et al 1937 Marley and Key 1963) It has the adult type of electrocortical activation by sensory stimuli and sympathomimetic amines produce behavioural and electrocortical alerting as in the adults (Marley and Key 1963)

The total brain 5 HT content discovered on the 25th 35th and 45th foetal days in the present investigation was very small and its rate of increase was slow on these days the cell bodies and nuclei in the cerebral cortex were growing fast. A rapid and statistically significant rise in 5 HT content occurred after that up to the 55th and 67th foetal days when the number and length of the cell processes increased sharply The brain 5 HT is located mostly in the nerve endings (Whittaker 1959 Potter and Axelrod 1962) and the development of the 5 HT level in the brain seems to be correlated with morphological development On the other hand developed electrocortical activity was found as early as the 45th foetal day when the whole brain 5 HT level was very low At birth the guinea pig is mature behaviourally and electrocortically but the 5 HT level in the brain is only half that of the adults 5 HTP decarboxylase activity remained at a low level relatively

slightly higher than 5 HT up to the 45th foetal day — and then it too rose significantly until full term though it is a soluble cytoplasmic enzyme (Bogdanski et al 1957) The development of MAO activity in the brain was different By the 25th foetal day it was already c 2/3 of the adult level and there was only a slight increase up to full term MAO is a mitochondrial enzyme (Weiner 1960) and mitochondria like cell bodies and nuclei may develop early so the rise in MAO activity might be correlated with morphological development The concentration of another mitochondrial enzyme, succinic dehydrogenase, rose in the guinea pig cortex from a low value to above the adult level between the 40th — 50th foetal days (Flexner et al 1953)

Since my first report (Tissari 1960) the brain 5 HT and associated enzyme contents in the developing guinea pig have been presented in two other studies Karki et al (1962) found that the 5 HT content was 77 %, 5 HTP decarboxylase 80 % and MAO activity 92 % of that of guinea pigs weighing 300—400 grams Smith et al (1962) studied 5 HT and 5 HTP decarboxylase on the 45th foetal day and at birth The 5 HT content on the 45th day was 50 % and at birth 70 % of the adult content and the 5 HTP decarboxylase activity 25 % and 60 % of the adult level respectively as in my material

In the rat and rabbit the critical period only occurs after birth The morphology of the rat cortex developed rapidly in the second postnatal week The behaviour and the motoric activity of 15 day-old individuals displayed a cortical level of neural correlation (Tilney 1933) Spontaneous and strychnine stimulated electrical activities were immature in the first week but after the 10th day they were adult in form (Crain 1952) In the rabbit brain the nerve cells and cell processes develop fast starting from the deeper layers between birth and the 15th — 18th day when the cytoarchitecture strongly resembles the mature structure The dendritic plexuses continue growing up to the 30th day particularly in the superficial layers (Schade 1959) Immature electrocortical activity is present from birth and becomes mature and indistinguishable from the adult pattern by the 10th — 15th day after birth (Schade 1959 Petersen et al 1964) An adult response to strychnine stimulation could only be registered about 20 days after birth (Bishop 1950)

The whole brain 5 HT content in the full term rat foetus was 20 % (Kato 1960) or 30 % (Karki et al 1962) of the adult content In the brain of the new born rat Nachmias (1960) found only c 1/3 of the adult content but other investigators have found approximately half (Karki et al 1962, Smith et al 1962 Tyce et al 1964) The 5 HT *content of the brain began to increase only in the second* week In these studies the content was roughly 60 % of the adult level at the age of 10 days during the critical period At the age of 15 days the 5 HT content of the rat hemispheres and brain stem measured separately were roughly 40 and 60 % of the adult levels (Kato 1960) 5 HTP decarboxylase in the rat brain was almost at the adult level at birth (Karki et al 1962, Smith et al 1962) but the MAO activity was only 25—30 % of the adults (Nachmias 1960 Karki et al 1962) and no increase occurred until the 10th postnatal day (Nachmias 1960)

The whole brain 5-HT content in the new born rabbit was 50 % of the adult level (Kärki et al 1962) The correlation between 5 HTP decarboxylase and MAO activity in the rabbit brain during development was similar to that found in the present work in the guinea pig brain 5 HTP decarboxylase activity in several

sites in the brain was about 30 % of the adult level in 3 day old rabbits and 70 % in 15 day old rabbits while the MAO activity was at the adult level 3 days after birth (McCaman and Aprison 1964)

In contrast to the findings in other species Pepeu and Giarman (1962) observed a higher brain 5 HT content in goat foetuses near full term than in the mothers the 5 HTP decarboxylase activity however was lower in the foetuses than in the mothers In these foetuses the blood 5 HT content too was 1 1/2 times the maternal content

Stacey and Young (1964) have recently studied the whole small intestine 5 HT content in 32—62 gram guinea pig foetuses The values they found particularly in their smaller specimens were considerably lower than those in foetuses of similar weight in the present material the relative 5 HT content at birth was c 60 % of the adult content, as against nearly 90 % in my material The divergence may partly be due to the difference in the 5 HT sources used In the present study a close correlation was found between the development of the 5 HT content and 5 HTP decarboxylase activity in the duodenum as well as in the brain 5 HTP decarboxylase activities were relatively somewhat greater than the 5 HT contents The development of MAO activity correlated poorly with that of 5 HT content in the intestine and this too resembled the results found in the brain It rose steadily during the whole foetal period

The circular muscle layer appears in the guinea pig intestine on the 24th foetal day and the longitunal muscle layer and the nervous elements on the 26th — 27th day The nervous elements then develop faster than the muscle layer and are adult in form by about the 50th foetal day Peristaltic contractions were first observed in isolated guinea pig intestine on the 26th — 27th day (Yanase 1907) Irregular peristaltic movements in vitro have recently been demonstrated in the gastrointestinal tract of the human foetus directly after the appearance of the circular muscle layer and before the development of the longitudinal layer and intramural nerves (Takita 1964) The commencement of propulsive activity in the digestive tract of the guinea pig foetus has been studied in situ by Becker et al (1940) The passage through the digestive tract of contrast medium injected into the amniotic sac was followed radiographically under conditions obviously corresponding to development in amnio Swallowing and gastrointestinal motility were found to start on the 42nd foetal day The rate of passage between different parts of the digestive tract increased with foetal age this increase was most rapid up to the 50th foetal day and then slowed down somewhat Spontaneous defecation and meconiophagy began on the 60th foetal day after which the whole cycle was repeated as many as five times before birth

The development of 5 HT and 5 HTP decarboxylase contents in the duodenum of the guinea pig observed in this investigation showed a striking correlation with the finding of Becker et al (1940) on the development of propulsive activity in the digestive tract of this species By the 45th day both the 5 HT content and the 5 HTP decarboxylase activity had risen from almost zero to c 40 % and over 80 % respectively of the adult levels The 5 HT content increased rapidly again before full term to almost 90 % of the adult level 5 HTP decarboxylase rose above the adult level No sharp changes were found in the duodenum MAO activity on the 45th foetal day by the 25th day some of the foetuses showed considerable activity and by full term the activity had reached the adult level

It is possible that the various components of the 5 HT formed — intracellular bound 5 HT 5 HT attacked by MAO and 5 HT released extracellularly — do not develop simultaneously in the intestine The 5 HT binding capacity of the enterochromaffin cells may develop early and the 5 HT formed become bound primarily to the intracellular granules (Baker 1958 Bertler et al 1960) In the third part of this study it will be demonstrated that the 5 HT binding capacity of platelets in full term guinea pig foetuses is well developed Bennett and Giarman (1964) could find no deficiency in the 5 HT binding capacity of the brain in either foetal or new born rats From the duodenum of the adult guinea pig we were able to extract a 5 HIAA content averaging 1 800 ng/g which in the full term foetus was roughly 60 % of the content in the mother (Tissari and Pekkarinen 1965) Since blood 5 HT is regarded as originating in the enterochromaffin cells of the intestine (Erspamer 1961a) it is probably correlated to the quantity of 5 HT released from them extracellularly In the blood of the full term foetuses I found only 30 % of the adult 5 HT level Thus despite the large stores of 5 HT in the intestine only a small quantity of 5 HT is released extracellularly and reaches mucosal sensory receptors impulses from which initiate the peristaltic reflex Some 5 HT however is released and may promote peristalsis The experiments of Bulbring and her co workers (Bulbring and Lin 1958 Bulbring and Crema 1958 1959a and b) and Lee (1960) (pp 12—14) made it seem probable that 5 HT plays a role in the peristaltic reflex But after depletion of intestinal 5 HT with reserpine (Bulbring and Crema 1959b) or a tryptophan deficient diet (Boullin 1964) peristalsis remained unchanged which suggests that 5 HT is not essential for the peristaltic reflex In the latter experiments the actual synthesis of 5 HT was probably stopped in all tissues Despite these reservations the very intimate correlation found in this study between the development of 5 HT and motoric activity in the intestine might be regarded as a sign of the physiological role of 5 HT in the peristaltic reflex The present data however are insufficient to exclude a general maturation of the intestinal metabolism at that time

Information on other species is scanty but points in the same direction In the intestine of the ox foetus small quantities of 5 HT and enterochromaffin cells appeared on the 30th day and showed a parallel increase up to the 70th — 80th foetal day after which they remained unchanged until the 220th foetal day This 5 HT content was about 1/4 of the contents in the calf and adult ox intestine presented in the same material (Faustini 1955) Enterochromaffin cells were first detected in the human foetal intestine between the 6th — 12th weeks and were adult in appearance (Cole and McKalen 1962) The human foetus begins to swallow *at the 5th month and the movement of the stomach musculature starts at the* 4th or 5th month (Windle 1940) while the muscular coat of the intestine is fully developed by the 5th month (Pollifrone 1952) In the intestine of the chick embryo the enterochromaffin cells appear on the 11th day of incubation (Simard and Van Campenhout 1932) and swallowing starts around the 9th day of incubation (Vrbitch 1924)

In the present study 5 HTP decarboxylase and MAO activities were found to develop slower in the kidney than in the other tissues examined Even on the 45th day the percentages were still very low compared with the adult level After that the activity of both enzymes increased rapidly to about 60 % of the adult level by full term The relative MAO activity values as presented in the section

of results are somewhat higher than the real values owing to the greater excess of substrate employed when examining the foetuses The development of 5 HTP decarboxylase and of MAO activity was more parallel in the kidney than it was in the other tissues studied

Very few data are available on the development of 5 HTP and MAO activity in the kidney of other species 5 HTP decarboxylase was absent in the kidney of full term foetuses and day-old rats After that it began to develop and reached the adult level by the age of one month (Huang et al 1960) The MAO activity in the kidney of the pig foetus rose steadily throughout foetal life and at full term it was still lower than in the adult (Weele and Hennig 1960) There was no MAO in the cortex of the kidney of a new born infant and in the medulla the activity was roughly 20 % of the adult level (Zeller et al 1940)

During prenatal life all species of mammals excrete urine into the amnion cavity but information on the kidney function of the foetuses is otherwise scarce and scattered The rate of urinary flow in pig foetuses at the beginning and middle of the foetal period was 20 and 15 times that of the adult (Perry and Stanier 1962) Kidney function tests on new born guinea pigs showed that the diluting and concentrating capacity of the kidney were already at the adult level pointing to maturity of tubular function but that water diuresis was slower than in the adult which suggested a deficiency in the glomerular filtration rate (Dicker and Heller 1951) On the other hand the kidney of the new born rat was entirely lacking in concentrating capacity and its water excreting capacity was lower than in the new born guinea pig the rat achieved adult type water diuresis at the age of one month (Heller 1951) The remarkably high 5 HTP decarboxylase and MAO activities that I found in the kidney of the full term guinea pig foetus and the lack of 5 HTP decarboxylase in the rat kidney at birth reported by Huang et al (1960) are related to the difference between these species in the maturity of the kidney at that time

The antidiuretic effect of 5 HT was demonstrated by Erspamer and Ottolenghi and by Sala and Castegnaro in 1953 However it is achieved with physiological doses only in the rat and mouse For other species unphysiological doses are needed (for references see Erspamer 1961b) In the experiments of Erspamer et al (1961) 5 hydroxy N acetyltryptophan and 5 acetoxy N-acetyltryptophan administered to rats did not cause antidiuresis even though these amino acids are changed enzymatically almost entirely in the kidney giving rise to high concentrations of 5 HTP and 5 HT there. Thus antidiuresis is caused only by 5-HT reaching the kidney via the bloodstream so it may be a question of 5 HT release into the plasma ensured by the platelets (Erspamer 1954a) Alkaline extracts of mammalian kidney were found to contain a substance capable of releasing 5 HT from platelets (Toh 1956) In the full term guinea pig foetus in which kidney function is very mature the blood 5 HT content in the present study was only some 30 % of that in the adult

In contrast to the other tissues studied I found high 5 HTP decarboxylase activity — almost 40 % of the adult level — in the liver as early as the 25th foetal day By the 45th day it had fallen slightly but it rose again to about 75 % of the adult level by full term It is difficult to interpret the significance of the early high 5 HTP decarboxylase activity in the liver It can hardly be associated with the formation of 5 HT for the total foetus 5 HT content on the 25th day is very low

It is possible that the various components of the 5 HT formed — intracellular bound 5 HT 5 HT attacked by MAO and 5 HT released extracellularly — do not develop simultaneously in the intestine The 5-HT binding capacity of the enterochromaffin cells may develop early and the 5 HT formed become bound primarily to the intracellular granules (Baker 1958 Bertler et al 1960) In the third part of this study it will be demonstrated that the 5 HT-binding capacity of platelets in full term guinea pig foetuses is well developed Bennett and Giarman (1964) could find no deficiency in the 5 HT binding capacity of the brain in either foetal or new born rats From the duodenum of the adult guinea pig we were able to extract a 5 HIAA content averaging 1 800 ng/g which in the full term foetus was roughly 60 % of the content in the mother (Tissari and Pekkarinen 1965) Since blood 5 HT is regarded as originating in the enterochromaffin cells of the intestine (Erspamer 1961a) it is probably correlated to the quantity of 5 HT released from them extracellularly In the blood of the full term foetuses I found only 30 % of the adult 5 HT level Thus despite the large stores of 5 HT in the intestine only a small quantity of 5 HT is released extracellularly and reaches mucosal sensory receptors impulses from which initiate the peristaltic reflex Some 5 HT however is released and may promote peristalsis The experiments of Bulbring and her co workers (Bulbring and Lin 1958 Bulbring and Crema 1958 1959a and b) and Lee (1960) (pp 12—14) made it seem probable that 5 HT plays a role in the peristaltic reflex But after depletion of intestinal 5 HT with reserpine (Bulbring and Crema 1959b) or a tryptophan deficient diet (Boullin 1964) peristalsis remained unchanged which suggests that 5 HT is not essential for the peristaltic reflex In the latter experiments the actual synthesis of 5 HT was probably stopped in all tissues Despite these reservations the very intimate correlation found in this study between the development of 5 HT and motoric activity in the intestine might be regarded as a sign of the physiological role of 5 HT in the peristaltic reflex The present data however are insufficient to exclude a general maturation of the intestinal metabolism at that time

Information on other species is scanty but points in the same direction In the intestine of the ox foetus small quantities of 5 HT and enterochromaffin cells appeared on the 30th day and showed a parallel increase up to the 70th — 80th foetal day after which they remained unchanged until the 220th foetal day This 5 HT content was about 1/4 of the contents in the calf and adult ox intestine presented in the same material (Faustini 1955) Enterochromaffin cells were first detected in the human foetal intestine between the 6th — 12th weeks and were adult in appearance (Cole and McKalen 1962) The human foetus begins to swallow at the 5th month and the movement of the stomach musculature starts at the 4th or 5th month (Windle 1940) while the muscular coat of the intestine is fully developed by the 5th month (Pollifroni 1952) In the intestine of the chick embryo the enterochromaffin cells appear on the 11th day of incubation (Simard and Van Campenhout 1932) and swallowing starts around the 9th day of incubation (Vrbitch 1924)

In the present study 5 HTP decarboxylase and MAO activities were found to develop slower in the kidney than in the other tissues examined Even on the 45th day the percentages were still very low compared with the adult level After that the activity of both enzymes increased rapidly to about 60 % of the adult level by full term The relative MAO activity values as presented in the section

5 HYDROXYTRYPTAMINE, 5-HYDROXYTRYPTOPHAN DECARBOXYLASE AND MONOAMINE OXIDASE IN GUINEA PIG TISSUES FROM BIRTH TO ADULTHOOD[1]

5 Hydroxytryptamine

The brain duodenum and blood 5 HT contents in 3 hour and 1 d v i guinea pigs were measured to study the effect of birth on the tissue 5 HT con i Although it was not possible to reckon the age of all the full term litters r copulation it can be concluded from the weights of the animals that t groups around the time of birth bore valid comparison The mean we ght full term foetuses was 93 g that of 22 3 hour old specimens 79 g and tl 1 day old guinea pigs 77 g Brain duodenum and blood 5 HT content also studied in 1 3 and 9 week old guinea pigs The mothers were ex simultaneously as adult controls

The results are presented in Table 8 The 5 HT contents of the ($P < 0.01$) duodenum ($P = 0.02$) and blood of the 3 hour old guinea pi about 40 % greater than those of the full term foetuses The brain 5 HT c at the age of 3 hours was 60 2 % of the adult level — the difference not significant — and that of the day old guinea pigs slightly less The bra n content of the young guinea pigs rose evenly between birth and adulth about 5 % a week At the age of 3 weeks the content was roughly 70 9 weeks 92 6 % of that of the adult controls (12 ± 3 month old females 5 HT content of the duodenum in the 3 hour old guinea pigs was 96 2 adult level In the day-old guinea pigs it was slightly less than in the 3 hour but there was no significant difference from the latter or from the adult mean duodenum 5 HT content of the 1 and 3 week old guinea pigs was the s In the 9 week specimens it was the same as in the 3 hour olds

The blood 5 HT content of the 3 hour old guinea pigs was 59 % of the adult level ($P < 0.05$) It increased more rapidly than the brain 5 HT content and was about 90 % of the adult level at the age of 1 week Then the blood 5 HT content almost doubled by the age of 3 weeks reaching 1 1/2 times the adult level In the 9 week olds it had fallen back to the level of the adult controls

The relative tissue 5 HT contents given in Table 8 have been calculated with respect to the mean value for the mothers of all the young guinea pigs When the brain 5 HT content of the foetuses of each group was expressed relative to

[1] A part of these result has already been communicated (Tissari 1960 1964 1965)

Table 9 5 HT content of platelets and whole blood platelet count in young guinea pigs and in female and male adults

Results expressed as mean values ± S E M Figures in parentheses represent number of measurements

Age	Platelet 5-HT content		Platelet count	
	5-HT ng/10⁹ platelets	% of adults[1]	Platelets/mm³ blood	% of adults[1]
1 day-olds	[2]16 0 ± 1 5 (8)	64 1	547 000 ± [illegible] 100 (8)	111
3 week-olds	[3]25 8 ± 2 5 (8)	103	598 000 744 000 (2)	136
6 week-olds	22 6 ± 2 5 (8)	90 2	510 000 ± 59 600 (6)	103
Adult females	25 0 ± 2 5 (10)		495 000 ± 66 000 (7)	
Adult males	22 6 ± 2 8 (5)			

[1] Relative values calculated with respect to the mean of adult females
The significance of the difference as compared with adult females is denoted by [2] = P < 0 01
The significance of the difference as compared with the preceding age group is denoted by [3] = P < 0 01

that of the mothers of the same group roughly the same percentages were obtained — for example 60 5 % in the 3 hour olds and 88 3 % in the 9 week olds The duodenum 5-HT contents in the adults varied considerably The relative contents compared with the mothers of the group were almost or entirely up to the adult level in every age group except the 1 day olds in which it was roughly 2/3 Neither did the blood 5 HT contents in the different adult groups vary much he content of the 3 week-olds was 178 % of that of their own mothers but this ifference was not significant (P < 0 1)

Owing to the considerable discrepancy found in the development of the 5 HT ontents of the intestine and blood, I also studied the platelet 5 HT content in a parate group of young guinea pigs using non pregnant adult females as con ols The results are shown in Table 9 The platelet 5 HT content in the day-old uinea pigs was 64 1 % of that of the adult controls (P < 0 01) In the 3 week old uinea pigs it was similar to the adult level and in the 9 week-olds slightly lower he whole blood platelet count in all the young age groups was similar to the dult level

ex Differences in Tissue 5 Hydroxytryptamine Contents

The most even distribution between females and males in this material was ound in the 3-hour 1 day and 3 week-old groups No differences between emales and males were found in the brain and duodenum 5 HT contents In the -hour and 1-day-old guinea pigs the blood 5 HT content was similar in females nd males in the 3-week-old males it was about 50 % greater than in the females hough this difference was not significant (Table 10)

As an adult comparison the 5-HT contents of the brain duodenum and blood ere studied in a group of 12 3 month-old males and non pregnant females

Table 10 5 HT contents of tissues of young female and male guinea pigs

Results expressed as mean values ± S E M Figures in parentheses represent number of measurements

Tissue and age	5-HT content ng/g or ng ml	
	Females	Males
Brain		
3-hour-olds	106 ± 14 2 (9)	100 ± 11 7 (11)
1-day-olds	102 ± 22 2 (6)	73 ± 7.3 (8)
3-week-olds	110 ± 9 1 (4)	124 ± 18 6 (6)
Duodenum		
3 hour-olds	13 900 ± 1 750 (10)	13 700 ± 1 150 (9)
1-day-olds	11 900 ± 2 850 (6)	11,300 ± 1 870 (7)
3 week olds	12 300 ± 1 810 (4)	10 200 ± 1 930 (6)
Whole blood		
1-day-olds	11 4 ± 3 5 (5)	13 6 ± 2 9 (3)
3 week-olds	14 5 ± 4 6 (6)	12.4 ± 1 1 (8)
3 week-olds	25 7 ± 5 6 (4)	37 6 ± 3.5 (7)

Table 11 5 HT contents of tissues of adult non-pregnant female and male guinea pigs

Results expressed as mean values ± S E M Figures in parentheses represent number of measurements

Tissue	5-HT content ng/g or ng/ml	
	Females	Males
Brain	149 ± 10 9 (7)	147 ± 9 2 (9)
Duodenum	13 400 ± 1 820 (6)	13 200 ± 1 390 (8)
Whole blood	29 0 ± 6 0 (9)	[1]12 0 ± 2 7 (9)

The significance of the differences between males and females is denoted by [1] = P < 0 05

that had last given birth not less than 2 months previously (Table 11) In the adults too no differences between males and females were found in the brain and duodenum 5 HT contents but the whole blood 5 HT content in the males was only 41 4 % of that in the females (P < 0 02) The material on the platelet 5 HT content (Table 9) also included a group of adult males No difference could be detected between males and females in platelet 5 HT content

Table 12 5 HTP decarboxylase activities of guinea pig tissues after birth

μg 5-HT formed/g of tissue/30 min

Age after birth	Brain 5-HTPD activity	Brain % of adults	Kidney 5-HTPD activity	Kidney % of adults	Liver 5-HTPD activity	Liver % of adults
1 week	13.3		2 860		1 140	
	14 4		3 000		1 110	
	16 7		2 350		1 000	
	15 0		3 160		952	
	9 8		3 000		741	
	10 7		3 000		1 000	
	11 5		3 000		867	
Mean ± S E M	13 1 ± 0 95	82 6	2 910 ± 99	107	973 ± 52	104
3 weeks	15 0		3 000		1 000	
	10 8					
	16 7					
	16 7		3 530		850	
	15 0		3 000		591	
	16 7		3 160		769	
	12.5		3 000		867	
Mean ± S E M	14 8 ± 0 88	93 5	[1]3 140 ± 102	116	815 ± 67	87 5
Adults	16 7		3 000		1 110	
	18 8		2 670		1 110	
	20 0		2 610		909	
	11 1		2 000		650	
	16 7		3 000		1 000	
	11 5		3 000		813	
Mean ± S E M	15 8 ± 1 5		2 710 ± 160		932 ± 74	

The significance of the difference as compared with the adults is denoted by [1] = $P < 0\ 05$

5-Hydroxytryptophan Decarboxylase

5-HTP decarboxylase activity was studied in the brain kidney and liver of 1 and 3-week-old guinea pigs Tissues from non pregnant females were simultaneously studied as adult controls The *duodenum was omitted from the postnatal* study because its 5-HTP decarboxylase activity had already attained the adult level in the full term foetuses and its postnatal 5 HT contents were fairly stable and resembled the adult level

The 5 HTP decarboxylase activity in the brain (Table 12) increased faster after birth than the 5 HT content. At the age of 3 weeks it was 93 5 % of the

Table 13 MAO activities of guinea pig tissues after birth

μg 5 HT metabolized/g of tissue/30 min

Age after birth	Brain		Kidney		Liver	
	MAO activity	% of adults	MAO activity	% of adults	MAO activity	% of adults
1 week	1 170		9 860		9,390	
	1 060		9 390		10 950	
	1 170		11 150		12 050	
	1 170		10 010		10 950	
	1 170		9 600		10 950	
	1 530		11 260		12 170	
Mean ± S E M	1 210 ± 66	99 2	10 210 ± 326	97 2	11 080 ± 130	95 4
Adults	1 170		9,390		11 730	
	1 060		9,390		11 310	
	1 170		11 150		12 050	
	1 170		10 640		10 950	
	1 390		11 020		10 950	
	1 370		11 430		12 670	
Mean ± S E M	1 220 ± 53		10 500 ± 367		11 610 ± 277	

adult level The rise in the 5 HTP decarboxylase activity in the kidney which had started towards the end of the foetal period continued strong after birth At the age of 1 week the value was above the adult level and at 3 weeks 16 % greater than the adult activity (P = 0 05) Liver activity also increased in the 1 week olds it had attained the adult level The activity found in the 3 week old guinea pig liver was slightly lower than this

Monoamine Oxidase

MAO activity was studied in the brain kidney and liver of week old guinea pigs and non pregnant adult females This activity too had reached the adult level in the duodenum before birth so the latter was not included in the postnatal study of MAO activity

Table 13 shows that the brain MAO activity of week old guinea pigs had attained the adult level There had been a rapid increase in kidney activity during the first week after birth — it had risen from about 60 % to the level of adult activity Liver MAO activity had also doubled in this time reaching 95 4 % of the adult level

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Fig 3 gives a summary of the changes in 5 HT contents and 5 HTP decarboxylase and MAO activities in tissues of young guinea pigs

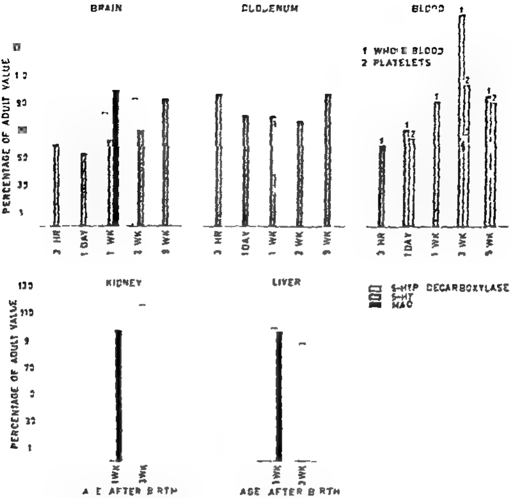

Fig 3 5-HT contents and 5-HTP decarboxylase and MAO activities in guinea pig tissues at various ages after birth expressed as percentages of the mean values found in adult tissues Values on horizontal lines refer to age after birth The values have been taken from Tables 8 9 12 and 13

Discussion

The brain duodenum and blood 5 HT contents in the 3 hour-old guinea pigs studied were about 40 % greater than in the full term foetuses Thus there were no abrupt changes coincident with birth The whole rat 5 HT content also rose evenly around the time of birth (Dixon 1959) On the other hand, the whole foetus 5 HT content in the mouse increased some 2 1/2 times on the day preceding birth (Robson and Senior 1964) Parratt and West (1957) found no rise in the 5-HT content of the guinea pig lung and rat lung and liver after birth at which time there was a transient increase above the adult level in their histamine content A

similar sharp rise in histamine content lasting for a few hours around the time of birth was noted in the whole rat and in several of its tissues (Dixon 1959)

I found that brain 5 HT content rose slowly and evenly after birth and in the 9 week old guinea pigs it was almost equal to that of the 12 ± 3 month old adult controls Brain 5 HTP decarboxylase activity had already risen to roughly the adult level by the age of 3 weeks when the 5 HT content was still only 70 % MAO activity was up to the adult level by the age of 1 week My results agree with those of Smith et al (1962) who found that the brain 5 HT content at 5 weeks and the 5 HTP decarboxylase activity at 3 weeks were almost up to the adult level in the guinea pig

The 5-HT content of the rat brain reached the adult level at the age of 40 days (Nachmias 1960) or somewhat earlier (Karki et al 1962) According to Kato (1960) the brain 5 HT content reached the adult level at 45 days in the male rat and only at 85 days in the female 5 HTP decarboxylase activity in the rat brain reached the adult level at birth (Karki et al 1962 Smith et al 1962) MAO activity 3 weeks after birth (Shimizu and Morikawa 1959 Nachmias 1960) In the rabbit 5 HTP decarboxylase activity in the cerebral cortex and caudate nucleus had not reached the adult level by the 32nd postnatal day but MAO activity was already a little below or above the adult level in several sites of the brain on the third day (McCaman and Aprison 1964)

In both the guinea pig and the rat the brain 5 HT content has only been found to attain its final level at adulthood almost concurrently with sexual maturity Kato (1960) found that administration of oestradiol increased the brain 5 HT content in young female and adult male rats Meyerson (1964) suggests that in the female rat there is a cerebral 5 HT ergic pathway that mediates heat reaction inhibition An increase in the 5 HT level in the brain brings about inhibition of the heat reaction while a decrease reduces it Robson and Botros (1961) found that 5 HT and MAO inhibitors administered to immature female mice inhibited the development of sexual maturity administered to mature female mice they reduced the weight of the sex organs

It should be noted that the present study of the development of the 5 HT content has been made on whole brain so changes occurring in certain areas or nuclei may not be brought to light In the rat the 5 HT content of the brain stem developed earlier than that of the hemispheres (Kato 1960) The 5-HT content of the rat pineal body achieved the adult level even at the age of 2 weeks (Quay and Halevy 1962) In the rabbit 5 HTP decarboxylase activity developed in the caudal regions of the brain faster than in the rostral regions (McCaman and Aprison 1964) MAO activity did not develop simultaneously in every part of the rat brain (Shimizu and Morikawa 1959) Another important point to bear in mind is that the majority of the brain monoamines are not essential for the transmitter function transmission depends on free and active monoamines that reach the receptors of the effector neurones (Carlsson 1964) Tyce (1964) reported that the rat brain 5 HIAA level between birth and 3 weeks was already up to the adult level

In Stacey and Young s (1964) material the whole small intestine 5 HT content of the guinea pig during the 40 days after birth varied over a broad range the mean being below the adult level In the present material the duodenum 5 HT content in 3 hour old guinea pigs was at the adult level Their stomachs all contained hay and milk coagula No 5 HT was found in guinea pig milk (Tissari

unpublished) The transition to extra uterine life and commencement of ingestion caused no changes whatever in the duodenum 5 HT content In the day old guinea pigs the duodenum 5 HT content was slightly lower 65 % of that of the simultaneous controls This observation appears to correlate with earlier findings that an increase in intraluminal pressure reduced the number of enterochromaffin cells in the intestine (Cole et al 1961) while in the rigid intestine the number and size of the enterochromaffin cells rose considerably (Pearse 1958) The duodenum 5 HT content of 9 week-old guinea pigs was fully adult in level

In the young guinea pigs the 5 HT contents of whole blood and platelet rich plasma were studied The 5 HT recovery from whole blood obtained by acetone extraction and bio assay has been reported as low 40 % (Hardisty and Stacey 1955 Rodnight 1958) 15 % (Toh 1954) In platelet rich plasma Hardisty and Stacey (1955) obtained over 95 % recovery and they considered the study of platelet rich plasma to be the most reliable method of blood 5 HT analysis I have not performed 5 HT recovery tests on either blood material The reproducibility of extraction however was very good with both materials Analysis of a series of nine duplicate whole blood assays revealed a mean deviation of 3 6 % from the mean of the duplicates and the standard deviation of the mean deviation was $\pm$ 2 3 % In a series of 23 duplicate platelet assays the corresponding figures were 3 1 $\pm$ 3 3 %

In both blood materials I found that the 5 HT content of day old guinea pigs was about 2/3 of that of the adults and in 9 week old guinea pigs it was slightly lower than in the adults In the 3 week olds however the values differed with both methods Their whole blood 5 HT content was 1 1/2 times the adult level while their platelet 5 HT content was equal to but did not exceed that of the adult *Another discrepancy was also found in results obtained using the two methods* in strictly paired tests the whole blood 5 HT content of adult male guinea pigs was under half the female content ($P < 0 02$) while their platelet 5 HT content was similar As the 5 HT extraction from both materials was equally reliable the discrepancies must have arisen from differences in the 5 HT content of the two sources

Mechanical trauma causes release of 5 HT from the platelets even repeated spinnings may lead to damage (Stacey 1958) MAO activity has been demonstrated in the platelets of several mammals (Paasonen et al 1964 Pletscher and Bartholini 1964) *To my knowledge no data have been reported in the guinea pig In* preliminary tests on blood 5-HT extraction I found that when guinea pig blood — collected in plastic tubes after severing the throat vessels — was left to stand at room temperature before acetone extraction its 5 HT content fell sharply 1/3—2/3 of it disappeared in 20—30 minutes When blood collected with a polythene catheter from the carotid artery was similarly allowed to stand no loss occurred within 60 minutes (Tissari unpublished) I assume that repeated spinnings in the preparation of platelet rich plasma may have led to 5 HT release so that slight physiological differences in content may have been eliminated The platelets of the guinea pig are possibly more susceptible to damage than those of some other species In any case I consider that immediate extraction of whole blood gives a reliable picture of the blood 5 HT content

Immediately after birth — as before — the relative blood 5 HT contents were much smaller than the relative duodenum 5 HT contents Between the ages of

3 hours and 1 day there was very little rise in blood 5 HT content though the chyme in the intestine increased considerably raising intestinal distension At the age of 3 weeks the whole blood 5 HT content was 1 1/2 times the adult level but there was no change in the duodenum 5 HT content. These findings were unexpected because blood 5 HT is considered to originate solely in the enterochromaffin cells of the intestinal mucosa (Erspamer 1954a 1961a) Stacey and Young (1964) too found that 5 HT in the guinea pig platelets appeared 10—15 days later and developed slower than the 5 HT content in the small intestine The total 5-OH indoles in new born rat blood were only some 10 % of the adult At the age of 10 days they had risen to about half the adult value and at 3 weeks they were up to the adult level (Tyce et al 1964) There are no data on the development of 5 HT in the rat intestine

In the guinea pig kidney and liver 5-HTP decarboxylase and MAO had reached the adult level at the age of 1 week in the present material After that, the 5 HTP decarboxylase activity in the kidney like the 5 HT content of the whole blood rose to above the adult level by the age of 3 weeks ($P = 0\ 05$) There is no precise information on weaning in the guinea pig Right from birth it eats other food besides milk Milk secretion appeared to continue for some 3 weeks after parturition In the rat weaning occurs at the age of 3 weeks at which time there has been shown to be a rise in the 5 HT and histamine contents of the skin and whole animal (Parratt and West 1957) Heart MAO too does not appear before this time (Novick Jr 1961) The increases noted in the guinea pig may be related to weaning

I could find no significant differences between female and male guinea pigs in brain and duodenum 5 HT contents at any age The whole blood 5 HT content of adult male guinea pigs was lower ($P < 0\ 02$) than that of females In the mouse (Albrecht et al 1956) and rat (Kato 1960) the brain 5 HT content was slightly higher in females than in males 5 HTP decarboxylase and MAO activities in the rat brain was significantly higher in females than in males (Skillen et al. 1961) But in the heart (Skillen et al 1962, Wurtman and Axelrod 1963) and liver (Wurtman and Axelrod 1963) MAO activity was significantly higher in adult male rats than in adult females

EFFECT OF MATERNAL 5-HYDROXYTRYPTOPHAN ADMINISTRATION ON FOETAL 5-HYDROXYTRYPTAMINE CONTENTS

The precursor amino acid, 5 HTP, was administered to pregnant guinea pigs to investigate the reason for the low tissue 5 HT contents found during foetal development This procedure was adopted because earlier tests had shown that 5 HT administered to the mother guinea pigs on the 25th and 67th days of pregnancy (2 mg/kg intravenously) did not enter the foetuses Despite the high 5 HT levels produced in the maternal blood the foetuses were alive during the 1 hour s observation time (Tissari unpublished) Some of the results of 5 HTP administration tests obtained in 67 day old foetuses have already been reported (Tissari 1964 1965)

Experiments on 67-Day-Old Foetuses

The 5 HT contents of the brain duodenum and whole blood were measured in the mothers and foetuses for 4 hours after administration of DL-5 HTP (20 mg/kg intravenously) to the pregnant guinea pigs at full term The results are shown in Table 14

30 minutes after 5 HTP administration the brain 5 HT content of the mothers had risen to over double that of the controls — similar pregnant females After 1 hour and 2 hours the content was slightly lower and after 4 hours it had fallen back to the control level 15 and 30 minutes after 5 HTP administration the brain 5 HT content of the foetuses was still at the level of the control foetuses but it only doubled in 1 hour ($P < 0\,01$) 2 and 4 hours afterwards it was still at the same level The relative rise of brain 5 HT content was similar in foetuses and adults but the absolute contents 1 hour ($P < 0\,05$) and 2 hours ($P < 0\,01$) after 5 HTP administration were smaller in the foetuses than in the mothers

Some of the individual values for duodenum 5 HT content found in the mothers after 5 HTP administration were larger than those normally obtained in this study The mean 5 HT content of all the mothers after 5 HTP administration was 18 900 ng/g which did not differ significantly from the mean for the control mothers 14 000 ng/g The duodenum 5 HT content of the foetuses had not increased the means being 9 000 ng/g in the control foetuses and 8 500 ng/g after 5 HTP administration

The 5 HT content of the maternal blood had risen about 3 fold 15 minutes

Table 14 5 HT contents of brain duodenum and whole blood in 67 day old guinea pig foetuses and mothers after maternal 5 HTP administration (70 mg DL 5 HTP/kg intravenously)

Results expressed as mean values ± S E M Figures in parentheses represent number of measurements

Tissue and age	Controls 5 HT ng/g or ng/ml	Minutes after 5-HTP administration 5 HT ng/g or ng/ml: 15	30	60	120	240
Brain						
Foetuses	[1]98 3 ± 7 6 (9)	122 ± 28 (3)	104 ± 27 (6)	[1]197 ± 26 (4)	[2]174 ± 26 (5)	199 ± 23 (5)
Mothers	141 ± 12 5 (12)		336 ± 37 (4)	267 ± 3 (3)	277 ± 5 (3)	204 139 (2)
Duodenum						
Foetuses	[1]9 000 ± 882 (9)	7 290 ± 1 6(0 (3)	6 310 ± 1 020 (6)	11 000 ± 1 100 (4)	[1]5 (10 ± 1 210 (5)	8 480 ± (00 (5)
Mothers	14 000 ± 1 940 (12)		24 000 ± 6 400 (4)	16 500 ± 3 870 (3)	10 700 ± 1 010 (3)	32 600 16 700 (2)
Whole blood						
Foetuses	[2]15 4 ± 2 0 (8)	65 5 ± 9 2 (3)	70 5 ± 8 4 (5)	139 ± 29 (4)	251 ± 48 (5)	2.0 ± 27 (4)
Mothers	28 1 ± 5 8 (9)	96 6 ± 12 8 (8)	137 ± 28 (10)	112 ± 16 (6)	136 ± 23 (4)	96 100 (2)

The significance of the differences between foetuses and mothers is denoted by [1] = P < 0 05 [2] = P < 0 01

Table 15   5 HT contents of platelet free plasma and platelets in 67 day old guinea pig foetuses and mothers after maternal 5 HTP administration (20 mg DL 5 HTP/kg intra-arterially 60 min previously)

Results expressed as mean values ± S E M Figures in parentheses represent number of measurements

Age	Controls		After 5 HTP administration	
	Platelet free plasma 5 HT ng/ml	Platelets 5 HT ng/10⁹	Platelet free plasma 5 HT ng/ml	Platelets 5 HT ng/10⁹
Foetuses	[1]0 38 ± 0 13 (4)	12.4 ± 2 0 (4)	33 5 ± 11 6 (10)	66 4 ± 5 6 (10)
Mothers	1 21 ± 0 26 (8)	15 2 ± 1 9 (8)	29 6 ± 7 8 (7)	76 7 ± 7 9 (7)

The significance of the difference between foetuses and mothers is denoted by [1] = $P < 0\ 05$

after 5 HTP administration It was at its maximum between 30 minutes and 2 hours afterwards being 4—5 times the level of the controls Four hours afterwards it appeared to have fallen a little The blood 5 HT content of the foetuses had also risen 4 to 5 fold as little as 15 minutes after 5 HTP administration and rose steadily until 2 hours after At 2 hours it was about 15 times the content of the control foetuses and almost twice that of the maternal blood At 4 hours it was still at the same level

No changes could be seen in the condition of the mothers after 5 HTP administration For one hour afterwards the foetuses were in good condition judging from the normal colour and pulsation of the umbilical vessels and reaction to pain At 2 and 4 hours some of the foetuses in the uteri opened earlier were dead At these times therefore hitherto intact litters were mainly studied and these were alive

Platelet and plasma 5 HT were studied in mothers and foetuses normally and 1 hour after 5 HTP administration (Table 15) Using the very sensitive assay method I was able to detect a minute 5 HT content in the plasma normally — mean 1 2 ng/ml in the mothers and 0 38 ng/ml in the foetuses ($P < 0\ 02$) After 5 HTP administration the 5 HT content of both mothers and foetuses rose to about 30 ng/ml There was no difference between the mothers and foetuses but the range of the values was much greater for the foetuses than for the adults For the 5 HT content of the maternal whole blood the mean and S E M were 105 ± 14 5 ng/ml and in two foetuses the contents were 177 and 203 ng/ml The condition of the foetuses after 5 HTP administration was good The method of foetal blood collection did not provide sufficient volumes of blood at later stages

Experiments on 25-Day-Old Foetuses

The above mentioned 5 HTP dose was administered to the guinea pig mothers on the 25th day of pregnancy and the 5 HT content of the whole foetuses was

Table 16 *5 HT contents of 25-day-old whole guinea pig foetuses and of whole blood of mothers after maternal 5 HTP administration (20 mg DL 5 HTP/kg intravenously)*

Results expressed as mean values ± S E M Figures in parentheses represent number of measurements

Age and tissue	Controls 5-HT ng/g or ng/ml	Minutes after 5-HTP administration 5 HT ng/g or ng/ml			
		15	30	60	120
Foetuses					
Whole body[1]	2.2 ± 0 22 (6)			124 ± 46 8 (7)	157 ± 18 7 (7)
Mothers					
Whole blood	43 2 ± 9 4 (7)	43 1 ± 3 1 (2)	143 ± 19 7 (6)	161 ± 20 6 (6)	193 ± 24 9 (3)

[1] 5-HT content measured without polyphenol oxidase treatment

assayed in 1 and 2 hours As can be seen in Table 16 even at this early stage of development the 5-HT content of the foetuses rose sharply after maternal 5 HTP administration The increase was about 15 fold compared with the whole foetus extract 5 HT content found after polyphenol oxidase treatment presented earlier The whole blood 5 HT content of the mothers was observed as a control and the findings corresponded to those above

Discussion

The 2 to 3 fold rise in the brain 5 HT content of the adult guinea pigs after 5-HTP administration and the fall of the 5 HT content to the preinjection level within four hours noted in this material corresponds to earlier observations (Davidson et al 1957 Paasonen and Giarman 1958) The increase in the brain 5 HT content of the foetuses began later and was still at its peak four hours later The relative increase was the same in the adults and foetuses and the maximum brain 5-HT content found in the foetuses after 5 HTP dosage was about 60 % of the maximum in the mothers The level of the 5 HTP transferred to the foetuses was of the same order as in the mothers, judging from the fact that, in addition to the blood 5-HT the kidney and liver 5 HT and 5-HIAA contents and the blood and duodenum 5 HIAA levels in the foetuses after 5 HTP dosage were as great as or greater than those in the mothers(Tissari and Pekkarinen 1965) The reason that the brain 5 HT content was lower in the foetuses than in the adults after 5 HTP administration may be an insufficiency of active transport of 5-HTP into the brain (Schanberg and Giarman 1960) or a deficiency of brain 5-HTP decarboxylase activity or 5 HT binding capacity The 5-HTP decarboxylase activity found 65 % of the adult level is probably already sufficient for it has been shown with the aid of specific 5-HTP/DOPA decarboxylase inhibitors (NSD compounds) that after almost complete decarboxylase inhibition the endogenous brain 5 HT and catechol amine levels remain unchanged (Brodie et

al 1962 Drain et al 1962) In the kidney and liver of full term foetuses too the 5 HTP decarboxylase activity was only about 2/3 of the adult level and as mentioned above the 5 HT and 5 HIAA contents after 5 HTP administration rose up to or above the adult level 5 HT formed in the brain became primarily bound because the simultaneous 5 HIAA content in the brain stem and hemispheres of the foetuses was only 1/3 of the adult content The 5 HIAA content normally found in the guinea pig brain was very low or not detectable at all and our material up to now does not permit comparison between adults and full term foetuses (Tissari and Pekkarinen 1965) After 5 HTP administration the foetus brain was evidently able to store a higher 5 HT content than the normal adult level and the low 5 HT content found seems primarily to be due to a deficiency of brain 5 HTP uptake

It is not known whether the transport of 5 HTP into the brain has any physiologial significance From indirect evidence it is assumed that the 5 HTP required to maintain the 5 HT level in the brain is produced locally (Erspamer 1961b) Tryptophan hydroxylation in vivo has been demonstrated by Gal et al (1964) in the pigeon brain and by Weber and Horita (1965) in the rat brain Gal et al calculated that synthesis of 5 HTP in the brain cannot account for all the cerebral 5 HT and that most of the 5 HTP needed must be supplied by other tissues The in vitro hydroxylation of tryptophan has recently been demonstrated by Grahame Smith (1964b) in the dog and rabbit brain

In conclusion the results presented suggest that the low 5 HT level normally in the brain of full term guinea pig foetuses may be due to lack of 5 HTP in the brain In the new born rat the low 5 HT level in the brain was assumed by Kärki et al. (1962) to result from a poor capacity to store and synthesize 5 HT Bennett and Giarman (1964) found that the new born rat was less able than the adult to synthesize cerebral 5 HT from L tryptophan administered intraperitoneally and assumed that its low brain 5 HT level is due to a deficiency of tryptophan hydroxylation

After 5 HTP administration in the rat (Davidson et al 1957 Erspamer and Bertaccini 1962) and guinea pig (Bulbring and Crema 1959b) low irregular increases in the intestinal 5 HT content were observed My findings on the duodenum 5 HT content of adult guinea pigs were similar In the duodenum of the foetuses too there was no increase in the 5 HT content after 5 HTP dosage For 5-HTP there is no active transport in the intestine (Cohen and Huang 1964) 5-HTP decarboxylase activity in the foetal duodenum was up to the adult level in the present study After 5 HTP administration in the guinea pig the 5 HIAA level in the duodenum rose multifold in both mothers and foetuses the foetal contents being somewhat greater (Tissari and Pekkarinen 1965) Thus after 5-HTP administration intensive 5 HT formation took place in the intestine but its stores were already normally full and the 5 HT formed was attacked by MAO or entered the blood In the full term guinea pig foetuses too the 5 HT stores were already normally full though the storage capacity was slightly less than in the adults

The blood 5 HT content of the adult guinea pigs rose 4- to 5 fold after 5 HTP administration as Udenfriend et al (1957b) had found in the dog The contents were still at the same level four hours later When 5 HT was administered to pregnant guinea pigs (2 mg kg intravenously) high blood 5 HT levels — c

10-fold — persisted for several hours (Tissari unpublished) After 5 HT is bound to platelets its half life in the platelets is very long (Udenfriend and Weissbach 1958) In adult plasma too I found 5 HT after 5 HTP dosage its content being some 30 % of the corresponding whole blood 5 HT content According to Udenfriend et al (1957b) the plasma 5 HT content was almost equal to the whole blood 5 HT content

The 5 HT content in full term foetus blood which normally had the lowest relative 5 HT level of the tissues examined rose to almost double the blood 5 HT content of the mothers after 5 HTP administration The increase began in the foetuses at the same time as in the mothers and the content was still the same four hours later The distribution of 5 HT between the platelets and plasma was similar to that in the adults The increase in blood 5 HT found after exogenous 5 HTP dosage evidently derives from all the extracerebral tissues containing 5-HTP decarboxylase After 5 HTP administration the 5 HT contents of the foetal kidney and liver were considerably higher than in the mothers the 5 HIAA levels in these tissues and in the foetal duodenum were slightly higher and in the foetal blood slightly lower than in the mothers (Tissari and Pekkarinen 1965) The higher than adult 5 HT contents in the blood of the foetuses are obviously due to deficient MAO activity in their kidney and liver compared with the decarboxylation efficiency as has been stated in the first part of this study

The present results show that the low blood 5-HT content normally found in full term guinea pig foetuses is not due to a deficiency of platelet 5 HT binding capacity Their platelet count was found to be equal to the adult level as was also noted by Stacey and Young (1964) at birth the percentage blood volume of guinea pigs was double that of the adult (Constable 1963) In the present study 5 HTP decarboxylase activity in its major sources was found to be sufficient to maintain the blood 5 HT at the adult level Thus the low 5 HT content in the blood must be due to deficient 5 HTP synthesis in the foetus

The assumption that the gastrointestinal tract is the major source of extra cerebral 5 OH indoles is based mainly on observed decreases in blood 5 HT contents and urinary 5 HIAA levels following the removal of large segments of intestine Haverback and Davidson (1958) in man and Rosenberg et al (1959) in man and dog found a clear decline in blood 5 HT and urinary 5 HIAA after massive resection of the gastrointestinal tract 5 HIAA excretion remained permanently low in colon extirpated patients (Bertaccini and Chieppa 1960) In the rat low 5 HIAA excretion persisted after removal of the large intestine after total gastroenterectomy it practically disappeared and the serum and spleen 5 HT contents decreased markedly (Bertaccini 1960) With the increase of enterochromaffin tissue in carcinoid patients the blood 5-HT content (Pernow and Waldenstrom 1954) and urinary 5 HIAA excretion (Page et al 1955) increased enormously The 5 HT content in the portal vein blood was greater than in arterial blood (Toh 1954) and that of the hepatic vein blood greater than in the blood from the vena cava (Erspamer and Testini 1959)

The small 5 HT quantities normally excreted in the urine (Twarog and Page 1953 Bumpus and Page 1955 Rodnight 1956) are considered to result from decarboxylation in the kidney (Rodnight 1956 Erspamer and Bertaccini 1962 Uuspaa et al 1962 1963) Uuspaa et al (1962 1963) found that the 5 HT content in the renal vein blood was significantly higher than the arterial blood content

while no differences were found in earlier studies on man and dog (Erspamer and Testini quoted by Erspamer 1954a) and on man (Bojs 1961) Uuspaa et al (1962 1963) assumed that normally the 5 HT formed in the kidney also enters the blood The source of the 5 HTP required is unknown since plasma contains no 5 HTP

Data on the occurrence of tryptophan 5 hydroxylase in peripheral tissues are also scanty Cooper and Melcer (1961) found the enzyme in the mucosa of the small intestine of rat and guinea pig and in small quantities in the kidney but other investigators (Renson et al 1962 Grahame Smith 1964a) have not yet been able to confirm their finding Tryptophan is a weak substrate for the phenylalanine-4-hydroxylase of rat liver (Renson et al 1962 Freedland et al 1961) but it appears to have no physiological importance in the biosynthesis of 5 OH indoles (Renson et al 1962 Freedland 1963) Weber and Horita (1965) found tryptophan hydroxylation in the rat intestine in vivo In carcinoid tumours Grahame Smith (1964a) also demonstrated in vitro hydroxylation of tryptophan a system which differed from the phenylalanine hydroxylating system of the rat liver

Presumably 5 HTP deficiency in some tissue is responsible for the low blood 5 HT levels in foetal and neonatal guinea pigs but it is difficult to say which Despite the almost adult 5 HT content found in the duodenum at full term and the fully adult content present from birth it is possible that total 5 HT synthesis in the intestine is still deficient The 5 HT formed may become primarily bound and thus the magnitude of the 5 HT stores possibly does not reflect that of real synthesis The duodenum 5 HIAA content in the full term guinea pig foetuses was about 60 % of the adult level (Tissari and Pekkarinen 1965) There may also be a deficiency in some component of the 5 HT formed which may remain excessive after intracellular binding or after being attacked by MAO and enter the blood The peak in blood 5 HT at the age of 3 weeks too may suggest that the 5 HT level in the intestine does not reflect the extent of true synthesis

It is notable that in the present study the development of 5 HTP decarboxylase activity in the kidney resembled that of the 5 HT content in the blood From the data now available it is not possible to decide whether this is only coincidental or whether the kidney is one of the sources of blood 5 HT

After 5 HTP administration to rats at different stages of pregnancy the whole foetus 5 HT content increased multifold (Davidson et al 1957) A similar situation was found in the 25 day old guinea pig foetuses in the present study It suggests effective 5 HT storage even at such an early stage since there was already MAO activity in the foetuses at this age

SUMMARY AND CONCLUSIONS

The appearance and occurrence of 5 HT and the associated enzymes 5 HTP decarboxylase and MAO during development was studied at their most typical sites in the guinea pig. The tissues analysed for 5 HT were the brain, intestine and blood. 5 HTP decarboxylase and MAO activities were measured not only in the brain and intestine but also in the kidney and liver. The measurements were started on the 25th foetal day and continued until adulthood or until adult levels were attained. The 5 HT contents of foetal tissues were also studied after administration of the precursor amino acid 5 HTP to mother guinea pigs.

The relative values of 5 HTP decarboxylase activity in the different tissues of the adult guinea pigs were: brain 1, duodenum 60, kidney 170 and liver 60; the absolute value of brain 5 HTP decarboxylase activity was 15.8 µg 5 HT formed/g of tissue per 30 min. The relative values of MAO activities in adult tissues were: brain 1 (corresponding to 1 220 µg 5 HT metabolized/g of tissue per 30 min), duodenum 7, kidney 9 and liver 10.

Brain. 5 HT appeared in the brain earlier than in any of the other tissues studied but reached the adult level last. On the 25th foetal day the brain revealed a mean 5 HT content of 16 ng/g, which was 10.9 % of that of the adult guinea pigs (about 12 months old). The 5 HT content of the brain remained at the same level up to the 45th foetal day. It then increased, being nearly doubled by the 55th foetal day, and again by the 67th foetal day (full term), when it reached 50.5 % of the adult content.

Birth caused no sharp changes in brain 5 HT content: in 3 hour old guinea pigs it was 60.2 % and in 1 day olds 53 % of the adult level. In the 3 week olds the content was roughly 70 % and in the 9 week olds up to the adult level.

The development of 5 HTP decarboxylase activity in the brain resembled that of the 5 HT content, but was slightly in advance. In the 25-day old foetuses it was 26.4 % of the adult activity, rising only after the 45th foetal day to attain 65 % of the adult level on the 67th foetal day. In the 1 week-old guinea pigs the brain 5 HTP decarboxylase activity was 82.6 % of the adult and at the age of 3 weeks it reached the adult level.

The development of MAO activity in the guinea pig brain differed. During the whole foetal period from the 25th day on, it was about 2/3 of the adult level. When studied one week after birth, it was identical with the adult activity.

When measured after maternal 5 HTP administration, the brain 5 HT content in full term guinea pig foetuses began to increase later than in the mothers. The maximum foetal brain 5 HT content after 5 HTP dosage attained 60 % of the

maximum maternal content which however is higher than the level normally found in adults The deficient brain 5 HT level of the full term guinea pig foetuses seemed to be due to lack of cerebral 5 HTP

The most rapid increase in brain 5 HT content occurred towards the end of the foetal period at the time when the nerve cell processes of the guinea pig cerebral cortex are developing rapidly (Peters and Flexner 1950) The brain 5 HT content was very low on the 45th foetal day when a continuous and well developed EEG is first obtainable (Jasper et al 1937 Flexner et al 1950) and only 60 % at birth when the guinea pig is mature behaviourally and electrocortically (Jasper et al 1937 Marley and Key 1963) If 5 HT has a transmitter function in the brain it is evident that the main part of it is not essential for transmitter function

Intestine The intestinal material studied consisted of pieces of duodenum removed in a standard reproducible way A very low 5 HT specific activity corresponding to 0 1 % and 2 1 % of the adult level could be detected in the duodenum of 25 and 35 day old guinea pig foetuses After this there was an almost 20 fold increase by the 45th foetal day at which time the amine reached 37 2 % of the adult content The 55th foetal day was the last time that the duodenum 5 HT content differed significantly from the adult level By full term there was a further significant rise in the duodenum 5 HT content which reached 86 1 % of the adult level

The duodenum 5 HT content in 3 hour old guinea pigs was at the adult level There were no significant changes in the duodenum 5 HT content of young guinea pigs observed up to the age of 9 weeks

In the duodenum too the development of 5 HTP decarboxylase activity ran parallel with but in advance of that of the 5 HT content Weak activity — c 1 % of the adult — was found in the intestine on the 25th foetal day After that it rose almost 100 fold by the 45th foetal day reaching 83 6 % of the adult activity The activity in 67 day old foetuses was above the adult mean

The sudden rise in 5 HT content and 5 HTP decarboxylase activity discovered on the 45th foetal day was not accompanied by a corresponding increase in duodenum MAO activity The latter rose evenly from 22 4 % on the 25th foetal day to the adult level at full term

After administration of 5 HTP to the mother guinea pigs at full term the 5 HT content of the duodenum did not rise either in the mothers or in the foetuses The capacity of the duodenum to store 5 HT was apparently less in full term foetuses than in adults but in the foetuses too the stores were already normally full

The development of duodenum 5 HT and 5 HTP decarboxylase showed a striking correlation with the appearance of gastrointestinal motility since Becker et al (1940) have demonstrated that in the guinea pig swallowing and gastrointestinal motility start on the 42nd foetal day Whether this finding is an indication that 5 HT plays a physiological role in the peristaltic reflex or only a sign of a general chemical maturation of the intestine at that time cannot yet be answered

Blood The blood was the last of the tissues studied to show an increase in 5 HT In whole blood of foetuses examined only on the 55th and 67th days the 5 HT contents were 20 8 % and 29 6 % of the adult level respectively

There were no sharp changes in the blood 5 HT content at birth The contents of 3 hour and 1 day old guinea pigs were 59 % and 70 2 % of the adult

level At the age of 1 week the content was approximately at the adult level and it increased to 1 1/2 times the adult level by the age of 3 weeks In 9 week old guinea pigs the whole blood 5 HT content had fallen again to the adult level

The blood 5 HT content of young guinea pigs was also studied using platelet rich plasma In the day old guinea pigs the platelet 5 HT content was about 60 % of the adult level and in the 3 and 9 week olds it was at the adult level the increase in whole blood content noted at the age of 3 weeks could not be detected by studying the platelets

After maternal 5 HTP administration the blood 5 HT content of the foet began to increase immediately as did that of the mothers but the increase in foetuses continued longer and attained almost twice the level in the m After 5 HTP dosage while the whole blood 5 HT content was about th foetuses and mothers the 5 HT content of the plasma rose from alm t roughly 30 ng/ml in both groups and the 5 HT content of the platelets w about the same in both

The finding that the development of intestine and blood 5 HT w chronous was unexpected since blood 5 HT is considered to originat intestine Possibly the magnitude of the 5 HT stores in the in estine d the true extent of biosynthesis there but the 5 HT formed may b primarily bound

Kidney A minute 5 HTP decarboxylase activity — 0 2 % of the a was found in the kidney on the 25th foetal day On the 45th day it w 4 3 % of the adult activity By full term there was a strong increase t reaching 60 4 % of the adult level In 1 and 3 week old guinea pig 5 HTP decarboxylase activity exceeded the adult level being signific in the 3 week-olds than in the adults

The development of MAO activity resembled that of 5 HTP d activity more closely in the kidney than in the other tissues In the 25 old foetuses the activity was roughly 7 % of the adult level There rapid increase up to full term when 62 6 % of the adult level was rea studied one week after birth the MAO activity in the kidney had adult level

The remarkably high activities of 5 HTP decarboxylase and M guinea pig kidney at birth were consistent with the advanced matur t pig kidney function at that time (Dicker and Heller 1951) It is possible that no causal relationship exists between the two events

Liver In contrast to the other tissues studied the liver was found have a remarkably high 5 HTP decarboxylase activity — 36 8 % of t by the 25th foetal day On the 45th it had decreased to almost half this v ut it had risen again by the 67th foetal day to 74 5 % of the adult level In the eek old guinea pigs the liver 5 HTP decarboxylase activity was as great in the adults A slightly but not significantly lower activity was found at the age of 3 weeks

MAO activity developed later than 5 HTP decarboxylase in the liver In 25 day-old foetuses it was roughly 10 % of the adult level and rose evenly up to full term at which time it had attained 59 % of the adult level 1 week after birth it was up to the adult level

Association with histamine formation is hypothesized to be of possible

significance for the high liver 5-HTP decarboxylase activity noted early in development. It is interesting to note, in regard to liver MAO, that considerable activity exists long before the start of oral ingestion.

[illegible]. The whole body 5-HT content and 5-HTP decarboxylase and MAO activities were studied at early stages of foetal life. The 5-HT content was quite low — c. 10 ng/g in 25-day-old and c. 15 ng/g in 45-day-old foetuses. On the 25th foetal day, a whole body 5-HTP decarboxylase activity corresponding to that of the adult brain was measured. The whole body MAO activity at this stage was roughly 50 % of the adult brain MAO activity.

One and two hours after maternal 5-HTP administration, the 5-HT content of whole 25-day-old foetuses was about 15 times greater than normal. This suggests effective 5-HT storage even at such an early stage.

Sex Differences in Tissue 5-HT Contents. When the tissue 5-HT contents of the foetal and young age groups were separated according to sex, no significant differences were found between males and females. In adults, there was no difference between males and females as regards the brain and duodenum 5-HT contents, but the whole blood 5-HT content of the adult males was only 41.4 % of that of the adult females, the difference being significant. In the platelet 5-HT content, no difference between the sexes could be detected.

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BY

TOIVO SUUTARINEN

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AND FROM THE ANESTHESIOLOGY DEPARTMENT, CLINICAL CENTER,
NATIONAL INSTITUTES OF HEALTH, BETHESDA, MD. U. S. A.

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To the memory of my father

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The experimental part of the present investigation was carried out at the Laboratory of Neuroanesthesia Surgical Neurology Branch National Institute of Neurological Diseases and Blindness and at the Anesthesiology Department Clinical Center National Institutes of Health Bethesda Md U S A where I had the opportunity to work during 1962—1963 The work was completed at the Children s Hospital University of Helsinki I wish to express my most sincere thanks to my chiefs and colleagues whose support helped me to accomplish this study

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Toivo Suutarinen

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Toivo Suutarinen

CONTENTS

I INTRODUCTION

Carbon dioxide is one of the basic requirements for life. There is little doubt that CO_2 is always bound up with the metabolism of the living organism, be it one cell or a large multicellular system.

That carbon dioxide plays a role in human physiology has long been recognized. Soon after its isolation in the middle of the 18th century its production in the process of respiration was established. The effect of carbon dioxide on the respiration and circulation was also discovered a long time ago, although it was assumed that CO_2 was an end product of irreversible metabolic reactions. The paramount and complicated physiological significance of this gas has been fully appreciated only during the last few decades.

Since the first studies concerning the effect of CO_2 on the circulation continuous interest has been shown in this subject. As early as 1915 Boothby had suggested that the hydrogen ion concentration of the blood had an effect not only on respiration but also on cardiac output. Other investigators (Liljestrand 1919) on the other hand found no changes in the circulation during CO_2 breathing.

Experiments concerning the circulatory effect of CO_2 were first made by giving various concentrations of CO_2 in room air during spontaneous breathing. The amount of CO_2 was expressed as a percentage of the air inspired. This method caused hypercapnia, but at the same time the oxygen content of the air was decreased and there was hypoxia as well. Soon pure oxygen was used to avoid hypoxia at higher concentrations of CO_2. Hypercapnia caused hyperventilation and these two independent phenomena together gave rise to variable results and consequent confusion.

Carbon dioxide was used in anaesthesia more than a century ago. In 1824 Hickman performed operations on animals using carbon dioxide for relief of pain (Lee 1959). Later CO_2 added to inspired gases was widely used by anaesthetists as a stimulus to respiration. The techniques used in anaesthesia also caused CO_2 accumulation and although the operation was well performed the patient was often lost owing to complications. The operative procedure had to be as short as possible and the good surgeon was a quick worker. Fundamental studies by several investigators called attention to the incidence of respiratory acidosis accompanying surgical anaesthesia (Beecher and Murphy 1950, Miller *et al* 1952). In view of these findings the trend latterly has been the elimination of carbon dioxide by hyperventilation. Hypocapnia, however, has its own adverse effects on the circulation, and in most modern anaesthetic techniques, *i e* using heart lung machines, CO_2 is added to the anaesthetic gases. In modern complicated anaesthetic techniques exact control of the physiological factors is essential and blood gas control is one of most important among them.

Sudden cardiac arrest or ventricular fibrillation has always been one of the main causes of death during an operative procedure This accident may be caused by hypoxia overdose of drugs and depression of respiration or peripheral vasomotor collapse Several observers have suggested that hypercapnia i e accumulation of CO_2 is one of the major factors responsible for this frightening occurrence (Young *et al* 1951 Miller *et al* 1952 Campbell 1953)

A vast amount of research has been carried out on the effects of CO_2 By now there is a voluminous literature dealing with the various experimental and clinical aspects of the circulatory effects of CO_2 The information relates to effects on heart rate and rhythm arterial and venous blood pressure cardiac output and the endocrine and nervous systems to mention only a few aspects However in studies dealing with the effects of CO_2 several difficulties are encountered Changes in blood CO_2 content always change the hydrogen ion concentration of the blood and cells as well and it may be quite difficult to separate these two factors The simultaneous and often antagonistic physiological effects of CO_2 on different organs complicate the individual response to CO_2 Anaesthesia has its own depressing effects on reflexes most anaesthetics abolish cardiovascular reflexes long before producing surgical anaesthesia Different experimental conditions and types of experiments various species of animals and variable instrumentation have their own effects on results Thus reported effects of acute hypercapnia on the cardiovascular system have varied During the last few years the development of delicate new instruments such as blood gas analysers (Lunn and Mableson 1963 McConn and Robinson 1963) and devices for continuous measurement of blood flow in intact arteries has provided many new possibilites for observing physiological phenomena in a functioning organism

Changes in arterial carbon dioxide tension are apt to occur when the thorax is opened and artificial ventilation instituted In the present study the aim has been to investigate cardiovascular reactions to changes in arterial carbon dioxide tension during light anaesthesia and thoracotomy

II REVIEW OF THE LITERATURE

A General effects of CO_2 on different body systems

Carbon dioxide is known to exert manifold effects on most body systems Fluctuations in the carbon dioxide tension bring in their wake a whole range of physiological reactions These are partly physico-chemical but they are also responses to stimuli transmitted from higher centres and systems

Carbon dioxide is one of the body s metabolic end products and regulatory controls should be able to secure normal body function even during disturbances in elimination of waste products

1 Respiration

The importance of carbon dioxide in respiratory physiology was already recognized by investigators in the eighteenth and nineteenth centuries However the quantitative regulation of respiration by CO_2 instead of oxygen was first clearly demonstrated by Haldane (Haldane and Priestley 1935 Perkins 1964) In 1893 experimenting on human subjects he was able to show that 3 per cent CO_2 in inspired air caused dyspnoea In 1905 Haldane and Priestley demonstrated the sensitivity of respiratory centre to the CO_2 pressure of the blood They showed that both the depth and frequency of respiration increased markedly during exposure to CO_2 and that hypocapnia was the cause of the apnoea commonly seen after hyperventilation (Haldane and Priestley 1905) Hypoxia on the contrary did not have any effect on respiration unless the oxygen in the inspired air fell below 12 per cent Hill and Flack (1908) experimenting on cats and dogs demonstrated the increasing excitatory effect of rising CO_2 on respiration An increase of CO_2 up to 35 per cent in inspired air progressively stimulated respiration but above 35 per cent CO_2 had a depressive effect They recommended the use of 5 per cent CO_2 as a respiratory stimulant in resuscitation Schneider and Truesdell (1922) studied the respiratory effects of increased CO_2 in man They observed that minute volume and tidal volume increased first the frequency being increased somewhat later These findings have since been confirmed by other investigators In 1947 Dripps and Comroe showed that 7 per cent CO_2 concentration produced nearly maximal effects on pulmonary ventilation in man

In respiratory control Haldane (Haldane and Priestley 1905) believed that the CO_2 level of the blood passing through the respiratory centre (whether carbon dioxide itself or the hydrogen ion concentration of the blood) regulated respiration In 1930–31 Heymans and his colleagues (Heymans and Neil 1958) published their studies in which they proved that there are peripheral chemoreceptors which also control respiratory function Changes in arterial pCO_2 provoke these carotid and

During changes in CO_2 tension pH and HCO_3^- are directly dependent upon the partial CO_2 pressure Thus it is practical to report these changes in arterial pCO_2 especially in the present study since the experiments were performed under conditions where a change in inspired CO_2 was the only variable affecting arterial pCO_2 and pH

3 *Central nervous system*

Central nervous system functions are strongly affected by abnormal concentrations of CO_2 In man chronic carbon dioxide accumulation causes various neurological symptoms including headache somnolence and disorientation (*c f* Autio ja Seppala 1964) In acute experiments either excitation or depression may occur depending on the concentration of CO_2 Woodbury *et al* (1958) and Woodbury and Karler (1960) divide the central nervous effects of CO_2 into three phases In the first phase there is decreased excitability which occurs with concentrations up to 20 per cent of CO_2 The second phase is characterized by increased cortical excitability so that seizures may even be seen This phase corresponds to 25—40 per cent CO_2 in the inspired gas Inhalation of more than 40 per cent CO_2 causes markedly decreased brain excitability and CO_2 anaesthesia This cerebral effect is similar to the anaesthesia induced by barbiturates or other anaesthetic agents Sudden removal of an experimental animal from a high CO_2 atmosphere to normal causes clonic seizures again (Woodbury *et al* 1958 Woodbury and Karler 1960)

Cerebrospinal fluid pressure is elevated during increased blood CO_2 concentrations In dogs an increase of up to 375 per cent above the control was observed in 10 minutes during diffusion respiration (Small *et al* 1960) Artificial ventilation with 5 10 and 15 per cent CO_2 showed similar results

Spinal cord function was depressed by inhalation of 8 9 per cent CO_2 (Kirstein 1951) A higher concentration may completely abolish spinal reflexes

Hyperventilation and the resultant hypocapnia may increase the irritability of the nervous system and muscles to such an extent that vigorous overbreathing may produce tetany (*c f* Brown 1953) Decreased hydrogen ion concentration is believed to be one of the main causes of this phenomenon

4 *Autonomic nervous and endocrine systems*

A large number of cardiovascular effects of CO_2 have their origin in the autonomic nervous and endocrine systems The mechanisms involved may be quite complicated nervous and hormonal stimuli and excitatory and depressant factors function simultaneously causing actions both centrally and peripherally

The immediate cardiovascular reaction to increased CO_2 tension is mediated through sympathetic stimulation (Goldstein and DuBois 1927 McArdle 1959 Wylie and Churchill Davidson 1960 Cross and Silver 1962) A rise in blood pressure increase in peripheral resistance and rise in heart rate have frequently been reported after a rise in CO_2 (Goldstein and DuBois 1927 McArdle 1959 Tenney 1960) However other simultaneous stimuli may exceed the sympathetic action the final result being a lowering in blood pressure and heart rate (Boniface and Braun 1953 Manley *et al* 1964)

The basic action of CO_2 is believed to be exerted on vasomotor centres in the medulla and on carotid and aortic receptors which then stimulate the sympathetic

nerves (Tenney 1960) However Downing *et al* (1963) were able to demonstrate a direct effect of CO_2 on the central nervous system They separated a dog s head from its normal circulation and denervated both the carotid sinus areas Then the brain was perfused with hypercapnic blood from another dog (pCO_2 above 100 mmHg) The result was a marked increase in blood pressure in peripheral vascular resistance and heart rate In this study it was obvious that severe hypercapnia of the central nervous system could elicit strong sympathetic responses

In a study made by Downing and Siegel (1963) the electrical sympathetic discharge to the heart was studied during hypoxia and hypercapnia Systemic hypercapnia increased the sympathetic discharge It was concluded that this increased sympathetic discharge was due to direct stimulation of centres within the central nervous system rather than reflex stimulation Parasympathetic cardioinhibitory centres on the other hand were stimulated reflexly by peripheral receptors This was confirmed in the study mentioned previously (Downing *et al* 1963) where separated central nervous system hypercapnia caused an acceleration in heart rate Sinus arrhythmia is a manifest sign of reflexogenic parasympathetic stimulation (Rushmer 1958 Best and Taylor 1961) Contrary to previous investigators however Young *et al* (1951) and Tenney (1960) reported that hypercapnia can cause central parasympathetic stimulation

CO_2 is probably also involved in the response to acetylcholine so that hyper capnia may prolong the action of acetylcholine Young *et al* (1951) in their experi ments on dogs observed that ventilation with 20 per cent CO_2 prolonged the cardiac standstill caused by vagal stimulation In another experiment where the vasodilator action of acetylcholine was measured this action was potentiated when the perfusing blood in the vessel was made hypercapnic (Tenney 1960)

A rise in arterial carbon dioxide tension results in release of catecholamine but only at high CO_2 concentrations in the inhaled gas In most studies the activation of catecholamine release could not be demonstrated at lower CO_2 concentrations which supports the evidence of direct early sympathetic stimulation by CO_2 Honig and Tenney (1957) could not show any difference in cardiovascular response to 6 per cent CO_2 breathing before and after adrenalectomy Tenney (1956) and Manley *et al* (1964) mentioned that CO_2 in inspired air did not have any effect on the adrenals if the percentage of CO_2 was below 15 per cent On the other hand a higher CO_2 concentration clearly caused catecholamine release from the adrenals and from other catecholamine depots (Tenney 1960) In cats adrenaline discharge could be demonstrated with CO_2 concentrations above 15 per cent In another report severe hypercapnia (15–30% CO) first markedly depressed the heart in both man and dogs The reflexly released adrenaline later restored heart function towards normal (Honig and Tenney 1957) Sechzer *et al* (1960) demonstrated that inhalation of CO_2 which raises end-expiratory CO_2 tension to 50–60 mmHg is accompanied by an increase in the plasma concentrations of adrenaline and noradrenaline These in turn cause a rise in heart rate cardiac output and blood pressure during hypercapnia Contrary to the above observations Morris and Millar (1962 a 1962 b) demonstrated that catecholamine liberation occurs in dogs even with mild or moderate hypercapnia so that an increase in catecholamines is an early response Non respiratory acidosis caused a similar catecholamine release

The mechanism by which CO_2 exerts its action on the sympatho-adrenal system is not quite clear Nahas (1956) and Bygdeman (1963) have stated that the effect is

primarily due to changes in hydrogen ion concentration rather than a direct CO_2 effect while the latter idea has been supported by others (Tenney 1956 1960)

Hypercapnia is known to inhibit the action of adrenaline and noradrenaline on the circulation (Tenney 1960 Bygdeman 1963) This effect apparently depends on changes in hydrogen ion concentration rather than directly on CO_2

Acute withdrawal of CO_2 causes a sudden rebound of the cardiovascular response This is probably a result of restoration of normal pH by rapidly decreasing CO_2 rather than of an acutely increased release of catecholamines The elimination of CO_2 is faster than the decrease in catecholamines and the result is a short term rebound overshoot (Tenney 1956 Millar 1960 Manley *et al* 1964)

Elimination of carbon dioxide is believed to have central nervous system effects opposite to hypercapnia (Burnum *et al* 1954) Vasodilatation as observed in certain vascular areas during hyperventilation is believed to be partly due to reduction of CO_2 at the vasomotor centre (Brown 1953)

Altogether changes in carbon dioxide tension activate a great number of responses With rising pCO_2 central and reflexogenic sympathetic stimulation is an immediate result followed by release of catecholamines from the adrenal medulla especially with higher concentrations of carbon dioxide Simultaneously central and peripheral parasympathetic activation takes part in the adjustment of the final response smoothing and stabilizing the reaction The responses observed in hypocapnia are mostly the opposite of the findings observed in hypercapnia

5 *Kidney and renal function*

Hypercapnia is known to result in changes both in renal circulation and in urine flow In a normal human subject inhalation of 10 per cent CO_2 caused renal vasoconstriction 13 per cent reduction in renal blood flow and 46 per cent increase in renal vascular resistance (Little *et al* 1949) Similarly vasoconstriction was observed in the human kidney during inhalation of 5 to 30 per cent CO in oxygen (Wylie and Churchill Davidson 1960) In dogs breathing 30 per cent CO_2 in oxygen caused marked reduction in renal blood flow (Stone *et al* 1958) and renal vasoconstriction (Best and Taylor 1961) Renal vasoconstriction which is observed during hypercapnia is apparently neurogenic in origin and seems to be associated with an increased release of catecholamines caused by high pCO_2 tension

While a sudden increase in arterial carbon dioxide tension causes marked reduction in urine flow and glomerular filtration rate the effect of chronic and moderate hypercapnia is remarkably slight In this case the renal mechanism tends to restore the normal acid base balance and the result is an acid urine and an alkaline reabsorbate (Kennedy 1960) With this in mind it is easier to understand how CO_2 breathing can cause water diuresis in man as was reported by Valtin *et al* (1959)

Hyperventilation frequently produces an increase in urine volume (Brown 1953) In dogs Emanuel *et al* (1957) observed an increase in renal arterial pressure vascular resistance and urinary output during hyperventilation.

6 *Biochemical changes*

It has been noticed that changes in pCO_2 may alter biochemical homeostasis In man metabolic acidosis was observed to follow hyperventilation by controlled respiration (Papadopoulos and Keats 1959) A rise in fixed acids and blood lactic acid

could be shown in the same study although these changes were not harmful Robinson (1961) also concluded that metabolic acidosis during passive hyperventilation in man is mild or negligible Similar metabolic acidosis in man with a rise in plasma citric and lactic acid connected with a fall in inorganic phosphorus was reported by Axelrod (1961) and with a rise in lactic and pyruvic acid by Eichenholz *et al* (1962)

Andersen and Svane (1962) studied biochemical responses in dogs during hyperventilation with various CO_2 concentrations Hyperventilation with 5 per cent CO_2 which caused hypercapnia and a rise in pCO_2 to 50—60 mmHg was followed by an increase in serum potassium and phosphorus while serum calcium was lowered Hyperventilation with 100 per cent oxygen leading to hypocapnia caused opposite changes Lactic acid concentration increased during the latter period

II Cardiovascular effects

The final haemodynamic response to variations in carbon dioxide tension is the balanced consequence of numerous actions in different body systems Various haemodynamic parameters may change quite differently and the final response of a certain organ depends greatly on whether an isolated organ or reactions of an organ in an intact body are subjected to study

1 *Isolated heart and vessels*

Isolated heart

In 1879 Gaskell (Gaskell 1880—1882) performed studies in which he examined the effects of alkaline and acid solutions on the isolated frog heart and arteries He observed that the effects of acid and alkali solutions were antagonistic in action When the acid solution had markedly weakened the force of ventricular systole the alkaline solution restored the force of the beat The smooth muscle of the smaller arteries reacted to dilute alkali and acid solutions in the same way as the cardiac muscle Jerusalem and Starling (1910) studied the effects of carbon dioxide on frog and tortoise heart in Ringer s solution saturated with a mixture of oxygen and CO_2 They reported that with moderate CO_2 tensions (2 to [illegible] per cent) the cardiac output increased but that a high percentage of CO_2 (12 to 20 per cent) diminished the output of the heart at each beat Experiments made by Williams (1955) also showed that the force of contraction was stronger in alkaline solutions and was weakened by acid He concluded that the force of contraction is directly dependent on pH This has later been confirmed by Price and Helrich (1955) and Price (1960 a)

Jerusalem and Starling (1910) also recognized the relation between CO_2 and heart rate In their experiment raising CO_2 concentration slowed the heart rate this slowing being the more marked the higher the percentage of CO_2 Whitehorn and Bean (1952) confirmed this finding In their studies on dog hearts 11 5 per cent CO_2 in O_2 at atmospheric pressure resulted in an initial acceleration of heart rate followed by a marked depression During artificial ventilation the acceleration phase was very brief bradycardia coming on very quickly The decreasing heart rate was connected with a depression in A V conduction Williams (1955) working on isolated rabbit auricles found that the frequency of the auricular beats was not affected by variations in CO_2 but was depressed when the acidity fell below pH 7 2 In his study the conduction velocity was higher during higher CO_2 concentrations Price and Helrich

(1955) also demonstrated slowing of the heart rate when ventilating lungs of a dog heart lung preparation with 5 per cent CO_2. The same was proved by McElroy *et al* (1958) on guinea pig hearts The cardiac rhythm however was not affected by pH changes even at pH values below 7 00 (Price and Helrich 1955 McElroy *et al* 1958 Price 1960 a)

Blood vessels

The effect of CO_2 on isolated or denervated blood vessels seems to resemble that on isolated heart

Gaskell (1880—82) had already been able to demonstrate the influence of acid and alkali solutions on vascular wall smooth muscle in isolated smaller arteries In a dog s denervated forelimb CO_2 inhalation caused vasodilatation (Etsten 1957) Fleishman *et al* (1957) came to the conclusion that decreased pH had a direct relaxing effect on vascular smooth muscle In his experiments the dilatation of arterioles was not so marked as that of postarteriolar vessels

The potent action of CO_2 on the cerebral circulation has long been accepted Kety and Schmidt (1946) clearly demonstrated the over all regulation of cerebral circulation by CO_2

Immersion of the human hand in CO_2 saturated water increased its rate of heat loss indicating a local vasodilatation exerted by CO_2 (Diji 1959)

Thus CO_2 seems to have a direct effect on blood vessels causing relaxation of vascular smooth muscle and is one of the very few metabolites having this action (Folkow 1960) Capillaries and veins are probably the vessels most affected (Price 1960 a) However in the intact animal or man the action of increasing CO_2 on the chemoreceptors and especially directly on the vasomotor centres in the medulla results a peripheral vasoconstriction (Best and Taylor 1961) This may be still further reinforced by release of catecholamines

Dilatation or constriction of veins can have a pronounced effect on cardiac output since veins contain up to 75 per cent of total circulatory blood volume (Albert 1963)

2 *Cerebral circulation*

Carbon dioxide has a very distinct role in the control of the cerebral circulation Carbon dioxide exerts a more powerful effect on the cerebral circulation than any other pharmacological agent or physiological condition can produce (Sokoloff 1959 1960) The inhalation of 5 per cent CO_2 in air increases the cerebral blood flow by 50 per cent and 7 per cent CO_2 more than doubles it (Kety and Schmidt 1948) On the other hand inhalation of 2 5 per cent CO_2 in room air has no effect on the cerebral blood flow in man, but 3 5 per cent CO_2 increases it by about 10 per cent This difference in percentage corresponds to 4 5 mmHg in arterial pCO_2 and seems to be the threshold for cerebral circulatory changes In dogs a change of 2 mmHg of arterial pCO_2 has been found sufficient to increase cerebral blood flow (Sokoloff 1960) Conversely decreased arterial pCO_2 causes cerebral vasoconstriction increased cerebral vascular resistance and decreased cerebral blood flow The blood flow during hypocapnia may be so reduced that cerebral ischaemia and syncope occur (Kety and Schmidt 1948)

The action of CO_2 on brain vessels is a direct tonically active vasodilatation causing a decrease in vascular resistance Hypocapnia has the opposite effect A secondary

result caused by the vasodilatation in increased intracranial pressure (Sokoloff 1959) Nervous control which takes part in most vascular functions probably plays no role in the control of cerebral circulation. The purpose of this exclusive cerebrovascular control of CO_2 is to maintain a constant pCO_2 in the brain

The cerebral vasoregulation is a direct carbon dioxide effect not connected with changes in pH Lambertsen *et al* (1961) compared changes in respiration and cerebral blood flow to alteration in blood pCO_2 and pH His conclusion was that brain blood flow was directly regulated by CO_2 because changes in pH produced by other means than changes in pCO_2 did not affect cerebral circulation

The close interrelation between carbon dioxide and cerebral circulation is well expressed in Sokoloff's (1960) statement » The relationship between CO_2 and cerebral circulation is so intimate and so fundamental that no physiological or pharmacological study of the circulation of the brain can be considered complete without the simultaneous examination of the effects of the experimental conditions on the state of CO_2 in the blood and cerebral tissue »

3 *Coronary circulation*

Information concerning the coronary circulation indicates a close relationship between this vascular bed and cardiac work An increase in the oxygen consumption of heart muscle or low oxygen tension in the blood cause marked coronary vasodilatation and an increase in coronary blood flow (Gregg 1960 1961 1963 Rushmer 1961)

Reports about the effects of CO_2 on coronary circulation are more variable Anrep (1936) concluded that CO_2 dilates coronary vessels Nahas and Cavert (1957) studying dog heart lung preparations found that in spite of severe myocardial depression the coronary sinus blood flow was well maintained during CO_2 ventilation if the pH was 7.25 or higher McElroy *et al* (1958) in their studies on isolated guinea pig hearts observed that the increased acidity caused by a rise in blood CO_2 tension increased the coronary blood flow They further observed that CO_2 did not have this effect when pH was kept constant and concluded that the change in pH caused by CO_2 variations was the actual factor responsible Feinberg *et al* (1960) concluded that hypercapnia caused an increase in coronary blood flow while high pH which is associated with hypocapnia apparently had no effect on coronary blood flow

Rowe *et al* (1962) studied the effects of hyperventilation on coronary haemodynamics in man and dogs In dogs the hyperventilation brought about passively with a respirator did not have any significant effect on coronary circulation. On the contrary active hyperventilation in man, leading to low pCO_2 tension was followed by a 30 per cent decrease in coronary blood flow

In conclusion Gregg (1960 1961) has suggested that in intact human subjects or animals neither increased pCO nor decreased pH affects the coronary circulation Rushmer (1961) signifies his agreement with this by stating that variations in CO_2 or pH probably have no effect on coronary vessels or coronary blood flow Finally Berne (1964) reviewing coronary blood flow regulation, concludes that the action of CO_2 on coronary blood flow is not quite certain. In any case it is very small as compared with the effects of CO_2 on the cerebral circulation

4 Myocardial contractility

In contrast to the isolated heart the myocardial contractile force of an intact animal appears to tolerate high carbon dioxide concentrations unchanged Nahas and Cavert (1957) who observed this in their studies, suggested that the simultaneously released adrenaline compensates the depression

Boniface and Brown (1953) using a strain gauge sutured on a dog s right ventricle could demonstrate a decrease in myocardial contractile force with 5 per cent CO_2 in inspired gas. An increasing concentration of carbon dioxide steadily lowered the contractile force A gradual return of contractile force to control level was observed when CO_2 was administered for 15 to 30 min

Monroe *et al* (1960) measured myocardial contractility by means of ventricular function curves Moderate elevations of pCO_2 (61—75 mmHg) did not depress contractile force but when the heart rate and left atrial pressure were kept constant these workers were able to demonstrate a consistent reduction in ventricular function Hypocapnia (pCO_2 6—13 mmHg) did not depress ventricular function

Previous finding were confirmed by Manley *et al* (1964) using the strain gauge arch previously described by Boniface and Brown (1953) They found a rapid reduction in myocardial contractile force when animals were exposed to 15 per cent CO_2 but the compensation of this reduction was also rapid In 10 minutes the force of myocardial contraction was back to 80 per cent of the control value

It seems evident that increase in carbon dioxide decreases the myocardial contractile force but simultaneous sympathetic stimulation and possible catecholamine release compensates this depression

5 Heart rate

Changes in CO_2 tension have marked effects on heart rate in men and animals Schneider and Truesdell (1922) studying men breathing CO_2 in air demonstrated an acceleration in heart rate even at one per cent CO_2 in inspired air Similarly Marshall (1926) observed an acceleration in heart rate during CO_2 breathing in man Goldstein and DuBois (1927) found that in man breathing 9 per cent CO_2 increased the heart rate by 18 beats per minute on an average Richardson *et al* (1961) also found a rise in heart rate during CO_2 breathing in man When arterial pCO_2 rose from 42 to 52 mmHg the average heart rate increased from 70 to 87 beats per minute

In dogs hypercapnia often causes bradycardia Marshall (1926) observed a decrease in heart rate in unanaesthetized dogs breathing 3—5 and 7—8 per cent CO_2 in air Miller *et al* (1952) found a marked bradycardia in dogs spontaneously breathing high concentrations of CO_2 in oxygen. Holmdahl (1956) observed a slowing of the heart rate during apnoeic diffusion oxygenation lasting less than 30 min He also pointed out the tolerance of the mammalian heart to acidosis Nahas and Cavert (1957) noticed marked bradycardia in dogs during artificial ventilation with 15 per cent CO_2 in oxygen Bilateral vagotomy did not abolish this bradycardia in open chest dogs anaesthetized with pentobarbital and ventilated artificially Feinberg *et al* (1960) observed a slight but not significant decrease in heart rate with *increasing* CO tension up to 10 per cent CO_2 in oxygen Manley *et al* (1964) observed a reduction in heart rate in artificially ventilated dogs during severe hypercapnia (15—30 per cent) of short duration in all the cases studied The heart rate did not recover from this decrease during CO_2 breathing This differed from changes in blood pressure

which after an initial decrease recovered in 10 minutes to near the control value Parasympathetic block with atropine 1 mg per kg intravenously did not abolish bradycardia during exposure to CO_2 In intact unanaesthetized dogs 5 and 7 per cent CO_2 in O_2 caused a progressive 20 per cent fall in heart rate from the resting value (Mithoefer and Kazemi 1964)

Downing *et al* (1963) studied cardiovascular responses to hypercapnia of the central nervous system in dogs by perfusing the central nervous system with hyper capnic blood They were able to demonstrate a marked increase in heart rate during increased CO_2 tension They concluded that severe hypercapnia of the central nervous system may elicit a strong sympathetic response and in this way play a significant role in circulatory homeostasis

The effect of hypocapnia on the heart rate in man was studied by Norlin (1932) Hyperventilation leading to hypocapnia was followed by an increase in heart rate Burnum *et al* (1954) found an increase of about 70 per cent in the heart rate of normal subjects when pCO_2 was lowered to approximately 20 mmHg by hyper ventilation Richardson *et al* (1961) could not demonstrate any significant change in heart rate in male volunteers when hypocapnia with a pCO_2 value of 25 6 mmHg was produced by hyperventilation On the contrary Thompson *et al* (1962) and Donevan *et al* (1962) demonstrated a marked increase in heart rate in normal subjects during voluntary hyperventilation

Voluntary CO_2 breathing seems to cause tachycardia in man while in anaesthetized as well as in artificially ventilated dogs increasing pCO_2 often causes bradycardia Separated hypercapnia of the central nervous system results in tachycardia Reports concerning the effects of hypocapnia are variable

6 *Arterial blood pressure and total peripheral resistance*

Blood pressure variations due to changes in inhaled CO_2 concentrations were observed during very early experiments Schneider and Truesdell (1922) in their studies on human subjects inhaling increasing percentages of CO_2 up to 8 per cent observed a continuous increase in both systolic and diastolic blood pressure The after-effect was a rapid fall in blood pressure Goldstein and DuBois (1927) observed a direct relationship between blood pressure and alveolar CO_2 tension in human subjects breathing 0 2 to 6 6 per cent CO_2 increasing CO_2 concentration always causing a rise in blood pressure Dripps and Comroe (1947) obtained similar results in their experiments 1–2 minute s inhalation of 30 per cent CO_2 in oxygen caused marked hypertension and bradycardia in conscious women (McArdle and Roddie 1958) This rise in blood pressure was progressive during inhalation of 30 per cent CO_2 (McArdle 1959) Systolic diastolic and pulse pressures increased significantly during inhalation of 7–14 per cent CO_2 in oxygen in every one of 12 healthy male volunteers (Sechzer *et al* 1960) Price in his review (1960 *a*) concluded that »arterial hyper tension is almost invariable during carbon dioxide inhalation»

Richardson *et al* (1961) studied circulatory responses to changes in blood CO_2 tension in human volunteers Inhalation of 7 per cent carbon dioxide in air for 7 minutes produced a marked increase in cardiac output blood pressure and heart rate ($p < 0.01$) Similar hyperventilation with CO_2 and without changes in arterial CO_2 tension did not cause any changes in these functions The authors concluded that circulatory changes during CO_2 hyperventilation are caused by carbon dioxide and

not mechanically by wider movements of the thorax during hyperventilation as previously believed (Grollman 1930)

Hyperventilation and subsequent hypocapnia can also affect arterial blood pressure During forced respiration in man Norlin (1932) observed only slight differences in blood pressure from resting values In experiments made by Kety and Schmidt (1946) on conscious young men the blood pressure did not fall in any case during active or passive hyperventilation but on the contrary tended to rise in most cases Burnum *et al* (1954) on the other hand found that the blood pressure fell in 33 out of 35 human subjects during hyperventilation of one minute s duration They suggested that hypocapnia had a direct action on the blood vessels causing over all vasodilatation and a fall in blood pressure Price (1960 a) also supported the idea that vasodilatation during hypocapnia is not of central nervous origin but is completely independent of nervous control Contrary to previous findings Richardson *et al* (1961) could not demonstrate any significant change in blood pressure or in heart rate during hypocapnia produced by hyperventilation

Total peripheral resistance mostly diminishes during CO_2 inhalation in man Generally the effects of hypercapnia on vascular resistance are relatively small and there is a reduction in calculated total peripheral resistance (Price 1960 a) Richardson *et al* (1961) observed a 23 per cent reduction in peripheral resistance during 7 per cent CO_2 inhalation and calculated that the total peripheral resistance was decreased during hypercapnia In different areas of the circulation elevation of carbon dioxide tension produces varying effects The vasodilatation of cerebral vessels by CO_2 is well known McArdle *et al* (1957) and McAardle and Roddie (1958) observed a striking increase in vascular resistance in the extremities during inhalation of 30 per cent CO_2 in oxygen

Hypocapnia produced by active voluntary hyperventilation with a 50 per cent reduction in arterial pCO_2 reduced systemic vascular resistance by 45 per cent (Burnum *et al* 1954) It was believed that the effect was a direct vascular effect of hypocapnia In their study Richardson *et al* (1961) suggested that respiratory alkalosis dilates forearm vessels and in this way increases blood flow

In animals studied mostly under anaesthesia and mechanical ventilation the results differ from those previously described in many ways The variability of preparations also greatly modifies the final results There are great individual and specific variations in the blood pressure response to CO_2 (Holmdahl 1956) Clowes *et al* (1955) studied the blood pressure response to 55 per cent CO_2 in oxygen The initial effect was a marked hypotension of short duration followed by a return near to control levels This was followed by a continuous gradual decrease Holmdahl (1956) observed a similar fluctuation in blood pressure during respiratory acidosis caused by apnoeic diffusion oxygenation in dogs After an initial fall there was a rise after 10 minutes of apnoea Nahas and Cavert (1957) again studying intact dogs paralysed with succinylcholine noticed no significant change in blood pressure during ventilation with 15 per cent CO_2 in O_2 Feinberg *et al* (1960) working with pentobarbital anaesthetized open chest dogs observed that increasing arterial CO_2 produced a slight but significant decrease in aortic blood pressure These findings were confirmed by Linde *et al* (1963) who found a slight fall in femoral artery pressure in dogs anaesthetized with pentobarbital under controlled ventilation with 5—8 per cent of CO_2 After adrenalectomy the fall in blood pressure was greater than in intact animals

Manley *et al* (1964) administering both 15 and 30 per cent CO_2 to dogs during controlled ventilation found a reduction in blood pressure which reached the lowest readings after approximately two minutes ventilation with CO_2. This was followed by a return towards the control level. After the termination of CO_2 the blood pressure rapidly rose to the control level or above it.

In man, inhalation of carbon dioxide often lowers total peripheral resistance. In dogs Itami (1912—13) observed that with respiration of 5 per cent carbon dioxide there was no marked change in the volume of the blood vessels. With 12 per cent CO_2 the rise of blood pressure was associated with vasoconstriction due to stimulation of the vasomotor centre. In intact animals, Clowes *et al* (1955) observed that moderate hypercapnia resulted in a peripheral vasoconstriction and elevation of peripheral resistance. Holmdahl (1956) observed increased total peripheral resistance during the first 10 minutes of apnoeic diffusion oxygenation. If the apnoeic period lasted longer the peripheral resistance diminished again. In rats anaesthetized with pentobarbital inhalation of 3 per cent CO_2 had no influence on circulation but 20 per cent CO_2 caused severe vasodilatation and a fall in blood pressure (Takács and Kálloy 1963).

Dowing *et al* (1963) in their studies on cardiovascular responses to ischemia and hypercapnia of the nervous system on dogs demonstrated that cerebral ischaemia produced a marked increase in blood pressure and peripheral vascular resistance. Hypercapnia prodused a similar but less marked response. It was suggested that hypercapnic blood perfusing the central nervous system markedly affects the systemic response to hypercapnia.

Passive hyperventilation and hypocapnia have long been known to cause a marked fall in blood pressure in anaesthetized animals (Kety and Schmidt 1946). The studies of Seevers *et al* (1940) showed the same phenomenon but also revealed that in unanaesthetized dogs hyperventilation did not cause any significant fall in blood pressure.

In man, carbon dioxide inhalation mostly causes a rise in arterial blood pressure while in animal experiments great variations due to experimental conditions are observed. In dogs a fall in blood pressure is often observed during hypercapnia but different findings are also reported. Observations on blood pressure during hypocapnia vary both in man and in dogs.

Total peripheral resistance mostly diminished in man during increased pCO_2, lowered TPR has also been reported during hypocapnia. In dogs increased TPR is mostly reported during hypercapnia, while hypocapnia seems to have the opposite effect.

7. *Cardiac output and stroke volume*

A quantitative method for determination of cardiac output was first described by Fick in 1870 (Fick 1870). However no great interest in the measurement of cardiac output was shown until the 1920's (Hamilton 1953). Since then, several different methods have been described for this purpose. These methods include the indicator dilution technique (Hamilton 1950, 1960, Fox and Wood 1960), pulse pressure method (Hamilton 1950), respiratory method (Hamilton 1950), ballistocardiography (Hamilton 1950, Rushmer 1961), radiocardiography (Hamilton 1960, Lewis *et al* 1962), radioisotopes (Braunwald *et al* 1962, Click *et al* 1962) and electromagnetic

blood flow measurement (Schenk *et al* 1958 Kolin 1960 Denison and Spencer 1960 Spencer and Denison 1960 Jochim 1962 a)

All the above mentioned methods have their advantages (Rushmer 1961) and draw backs (Miller *et al* 1962 a Sleeper *et al* 1962 Marshall *et al* 1962) All but the electromagnetic technique can be used without surgical preparation Electromagnetic measurement requires surgical exposure of the vessel and positioning of the probe around the vessel involved Under good conditions this technique provides a continuous quantitative recording of mean and pulsatile flow with an accuracy of ± 5 per cent (Jochim 1962 a) Compression of an artery by 20 per cent with the probe only changes the mean blood flow very slightly (Spencer and Denison 1960) At the present the electromagnetic flow technique is superior to the other flow measuring methods (Wetterer 1963)

Cardiac output is the total result made up of a combination of several different factors The two main components are heart rate and stroke volume which together regulate the amount of blood pumped in a fixed time Under normal conditions heart rate is controlled by the sinoauricular node which in turn is influenced by sympathetic and parasympathetic impulses *i.e* by higher cardiovascular regulatory centres Stroke volume is dependent on the amount of blood the heart can take in on each diastole and the amount of blood it can eject during systole against the pressure in the arteries A certain variable amount of blood is left in the ventricle at the end of each systole (Rushmer 1961)

Heart rate and storke volume are independent of each other Quite often increased heart rate is associated with decreased stroke volume and decreased cardiac output (Berry *et al* 1958 Rushmer 1961) In adjusting changes in cardiac output it is often difficult to say whether the change is due to elevated stroke volume or more rapid heart rate (Horwath and Howell 1964)

The relationship between increasing heart rate and stroke volume and cardiac output was studied on thoracotomized dogs by Berglund *et al* (1958) Heart rate was controlled by electrical stimulation It was noticed that stroke volume was always greater at lower frequencies and decreased progressively with rising rate Cardiac output increased with rising rate up to a heart rate of 90/min was then at a maximum between 90 to 180 beats/min and decreased again when the rate rose above 180 min

Warner and Toronto (1960) carried out similar studies controlling heart rate by direct electrical impulses They found no relationship between the heart rate and cardiac output Stroke volume was highest with slow rates and decreased as the rate became faster Miller *et al* (1962 b) also examined the effect of controlling heart rate on cardiac output in dogs When the rate was increased from 30 to 60/min the cardiac output rose markedly Between 60 and 90 min the cardiac output increased still further remaining constant at rates between 90 and 150/min If the rate rose above 150/min the cardiac output tended to fall Stroke volume was highest at rates below 60/min and with increasing heart rate the stroke volume decreased progressively

In spite of inaccurate methods of measuring cardiac output, the effect of CO_2 on cardiac output was widely discussed in the beginning of this century Many investigators believed in the stimulating effects of carbon dioxide However Liljestrand (1919) demonstrated that an increase in alveolar CO_2 tension high enough to cause forced breathing in man did not markedly influence cardiac output. It appears possible however that the increase in alveolar CO_2 was not high enough to increase

arterial pCO_2 during hyperventilation Marshall (1926) could not demonstrate any influence of CO_2 on cardiac output in unanaesthetized dogs breathing 3—5 per cent CO_2 in air while concentrations of 7—8 per cent CO_2 decreased the cardiac output St Janousek (1929) reported that inhalation of CO_2 up to 4 per cent decreased cardiac output while higher concentrations caused an increase Grollman (1930) stated that in man inspiration of carbon dioxide in air has no effect on cardiac output until the CO_2 concentration in the inspired air exceeds 6 per cent With higher CO_2 concentrations the cardiac output is increased

In 1943 Asmussen showed that ventilation and cardiac output increased together in normal men breathing CO_2 in air in the supine position In a tilted position at an angle of 60 no increase in cardiac output was observed The author concluded that the increase in cardiac output during CO_2 breathing was due to mechanical factas of hyperventilation and not to a chemical action of CO_2 However Burnum *et al* (1954) and McGregor *et al* (1962) demonstrated that when arterial pCO_2 was kept constant hyperventilation with CO_2 did not increase cardiac output This indicated that it was increase in pCO_2 and not in ventilation which elevated cardiac output during CO_2 breathing The effects of CO_2 may be mediated through central nervous and sympatho-adrenal systems to the heart Other recent reports concerning changes in cardiac output during hyperventilation with increased alveolar CO_2 mostly demonstrate an increase in cardiac output in man (Price 1960 a Wade and Bishop 1962)

Richardson *et al* (1961) measured the effect of 7 per cent CO_2 on the cardiac output of male volunteers When the average rise in arterial pCO_2 was from 42 to 58 mmHg cardiac output rose in every subject attaining a mean value of 45 per cent greater than the controls Similar hyperventilation with constant arterial pCO caused only insignificant changes in circulation Decreased pH without changing pCO_2 had no effect on cardiac output The author concluded that elevation of pCO_2 rather than a fall in pH was the factor affecting cardiac output during CO_2 breathing McGregor *et al* (1962) studied the influence of hyperventilation on cardiac output in 7 normal subjects in the supine position using the dye dilution method The study was divided into three groups The first was voluntary hyperventilation with room air followed by hypocapnia The second was hyperventilation with a CO_2-air mixture so that the alveolar carbon dioxide content was at a normal level Finally 5—10 per cent CO_2 was added to the inspired air the result being hypercapnia in spite of hyperventilation Voluntary hyperventilation with air leading to hypocapnia invariably increased cardiac output During voluntary hyperventilation and normal pCO_2 there was a smaller increase in cardiac output In a third hypercapnic group there was no change in the first two minutes but thereafter the cardiac output was increased The authors suggest that the response to hyperventilation is mainly determined by the CO_2 content of the inspired air

Auld *et al* (1962) drew the opposite conclusion from their results They measured cardiac output in children anaesthetized with 50 per cent O_2—50 per cent N_2O and halothane At first the children breathed the anaesthetic mixture alone and then mixed with 2 4 or 6% CO_2 The pCO_2 readings varied from 29 mmHg to 80 mmHg No clear change in cardiac output heart rate or peripheral resistance could be demonstrated The authors concluded that the increase in cardiac output during CO_2 hyperventilation is related to respiratory movements rather than to a rise in CO_2

Similarly hypercapnia often increases cardiac output in dogs Paulet and Bernard (1961) noticed a 40 per cent increase in cardiac output in intact anaesthetized dogs during ventilation with 12 per cent CO_2 In intact anaesthetized dogs Linde *et al* (1963) reported a 21 per cent increase in cardiac output when arterial pCO_2 rose from 35 mmHg to 63 mmHg (mean values of 8 experiments) Here again, the type of respiration greatly affects the cardiac output Linde *et al* (1961) noticed that with positive pressure breathing cardiac output fell significantly in dogs anaesthetized with sodium pentobarbital (25 mg/kg) as compared with values measured during spontaneous respiration

All in all reports concerning the effect of changing pCO_2 on cardiac output are variable and in part inconsistent in human as well as in animal experiments Often hypercapnia is seen to result in an increase in cardiac output but similarly an increase in cardiac output is seen during hypocapnia Differences in experimental conditions concentration of carbon dioxide ventilation etc probably affect the observed results considerably leading to variable conclusions

8 *Right atrial pressure*

Right atrial pressure is an important component in the regulation of cardiovascular function and cardiac output Right atrial pressure does not directly determine the cardiac output but is related to changes in this function (Guyton 1955) Right atrial pressure also has a very marked effect on venous return to the heart The higher the right atrial pressure the higher is the backpressure to the venae cavae which can prevent blood from returning to the heart (Guyton 1963)

The change in right atrial and central venous pressure elicits reflexes which affect cardiovascular function In the Bainbridge reflex it is believed that the rise of pressure in the right atrium and venae cavae initiates a mechanism which causes tachycardia (Bainbridge 1915 Corday and Irving 1961 Aviado 1962) Other investigators in subsequent attempts to confirm this reflex have obtained variable results It was shown that instead of tachycardia increased filling of the right atrium produced bradycardia and hypotension (Neil 1962) On the other hand tachycardia provoked with atropine produced an increase in cardiac output and a decrease in mean central venous pressure (Berry *et al* 1959)

Schneider and Truesdell (1922) measured venous pressure during CO_2 breathing in man At 7 per cent CO_2 the venous pressure was increased by 74 per cent on an average In some experiments as little as 1 per cent CO_2 caused an increase in venous pressure

On intact animals paralyzed with Anectine Nahas and Cavert (1957) found an increased central venous pressure during ventilation with 15 per cent CO_2 in oxygen. This rise became evident after 10—15 minutes administration of CO_2 and was not abolished by vagotomy In the heart lung preparation, 15 per cent CO_2 caused a marked elevation of right atrial pressure in less than 3 minutes accompanied with acute myocardial failure A shift to 5 per cent CO_2 abolished cardiac failure and right atrial pressure returned to the control baseline

Montemartini *et al* (1962) measured venous pressure during hypoxia as such and during hypoxia with 2 per cent CO_2 in man Hypoxia elevated venous pressure slightly but during CO_2 breathing the increase in venous pressure was more marked An increase in venous pressure during hypercapnia was also reported by Seveso (1962)

A fall in venous pressure has commonly been found after voluntary hyperventilation (Brown 1953) Price (1930 a) also mentioned that hyperventilation may augment venous return to the heart but this does not necessarily increase cardiac output Thompson *et al* (1962) noticed a fall in mean central venous pressure during voluntary hyperventilation in man Rowe *et al* (1962) found that active hyperventilation caused a reduction in right atrial pressure in man but in dogs with closed chest passive hyperventilation brought about a significant increase in right atrial pressure The difference was believed to be due to the difference in the method of ventilation

Miller *et al* (1962 b) studied the effect of increasing heart rate on cardiac output They noticed elevated right atrial pressure at low (below 60/min) and high (above 150/min) rates This could have been due to venous pooling or increased venous tone

In most reports CO_2 breathing has increased central venous or right atrial pressure both in man and in animals but often the CO_2 concentration used has been very high Hypocapnia produced by hyperventilation often causes a reduction in central venous and right atrial pressure but differences due to experimental conditions are seen

9 *Cardiac rhythm and sinus arrhythmia*

A moderate rise in arterial pCO_2 is very rarely followed by arrhythmias although higher carbon dioxide concentrations may cause abnormalities in ECG In conscious men a pCO_2 level twice the normal had no remarkable effect on the cardiac rhythm (Price 1960 a) Changes occasionally observed during hypercapnia include ST elevation or depression (Miller *et al* 1952 Holmdahl 1956 Manley *et al* 1964) prolongation of P R interval and changes in T waves (Holmdahl 1956 Price 1960 a Seveso 1962) Ventricular or nodal extrasystoles and accentuation of sinus arrhythmia have also been reported (Sechzer *et al* 1960 Price 1960 a) One explanation of irregularities in rate and rhythm may be the stimulation of the sympatho adrenal system and release of catecholamines which is seen during severe hypercapnia (Price 1960 a) On the other hand Sealey *et al* (1954) concluded that increased carbon dioxide tension in itself provokes cardiac slowing and may lead to asystole

However sympatho adrenal stimulation combined with release of catecholamines high CO_2 tension or respiratory acidosis are not the only factors leading to arrhythmias during hypercapnia Young *et al* (1951) Clowes *et al* (1955) and Price (1960 a) suggest that hypercapnia by itself does not directly lead to bradycardia or cardiac arrest but exaggerates the cardiac response to vagal stimulation Thus cardiac sensitivity to vagal stimulation is higher during hypercapnia than under normal conditions

Several investigators have pointed out the high incidence of severe arrhythmias during the posthypercapnic period Itami (1912—13) observed ventricular fibrillation in cats and dogs after cessation of CO_2 breathing Brown and Miller (1952 a) reported ventricular fibrillation in 11 out of 15 dogs when alveolar carbon dioxide tension was rapidly lowered after 4 hours of breathing 30—40 per cent CO_2 in oxygen Brown and Mowlem (1960) demonstrated a significant elevation of coronary sinus plasma potassium above the potassium concentration in the aorta during the immediate posthypercapnic period This difference in potassium concentration was believed to indicate potassium loss from the heart and was accompanied by cardiac irregularities Andersen and Svane (1962) on the other hand observed a rise in plasma potassium

during hyperventilation with 5 per cent CO_2. A sharp decline in plasma potassium was observed during the recovery period.

In most previous studies, however, 30—40 per cent CO_2 was given for several hours, a situation which far exceeds any clinical conditions. McArdle (1959) studied electrocardiograms in man during and after inhalation of 30 per cent CO_2 in oxygen for 1 to 2 minutes. Auricular extrasystoles in 12 out of 30 records was the only abnormality observed in this study. In other studies on man where pCO_2 was approximately 75—200 mmHg, cardiac arrhythmias were rare during or immediately after CO_2 breathing and all irregularities disappeared in 5 minutes after termination of CO_2 inhalation (Price 1960 a, Sechzer *et al.* 1960). No fibrillation was observed in these studies. The tolerance of the dog heart to carbon dioxide has been similarly demonstrated. In a study made by Brown and Miller (1952 b), the inhaled carbon dioxide concentration was gradually increased and in five out of seven dogs it reached levels above 90 per cent before cardiac arrest followed. Holmdahl (1956), studying dogs and rabbits, was unable to bring about any serious ECG disturbances by vagal stimulation after 45 minutes' apnoeic diffusion oxygenation with associated severe respiratory acidosis. The results during the posthypercapnic period were the same. The tolerance of the heart to severe hypercapnia was also noticed by Manley *et al.* (1964). In their studies no arrhythmias were seen during the ventilation of 15 or 30 per cent carbon dioxide in oxygen in pentobarbital anaesthetized dogs, nor could arrhythmias be observed during the immediate posthypercapnic period.

The S-A node has been shown to be more sensitive to changes in vagal tone than to altered sympathetic activity (Rushmer 1958, 1961) so that changes in vagal activity may lead to changes in cardiac rhythm. These changes are mostly observed as abnormalities in heart rate. Sinus tachycardia, sinus bradycardia and sinus arrhythmia are the commonest in this category. Sinus arrest (sino-auricular block), an interruption in S-A node function leading to loss of a complete beat, is also vagal in origin (Lipman and Massie 1956, Best and Taylor 1961, Corday and Irving 1961, Rushmer 1961).

Sinus arrhythmia is a rhythmic irregularity in the heart rate caused by phasic variation in vagal tone. Mostly this is initiated by respiratory activity (Widdicombe 1954, Clynes 1960, Corday and Irving 1961, Rushmer 1961). Impulses from the S-A node vary with the respiration, causing acceleration of heart rate towards the end of inspiration and slowing at the end of expiration (Hering-Breuer reflex) (Hering 1868, Breuer 1868, Widdicombe 1963, Burch 1963). The elimination of sinus arrhythmia by atropine or vagotomy indicates the vagal origin of this reflex (Widdicombe 1963).

Increased vagal tone or intense vagal stimulation can momentarily interrupt S-A node action completely, leading to sinus arrest and a prolonged pause between two normal heart beats. Stimulation of the carotid sinus or deep inspiration have been noticed to initiate this abnormal heart action (Rushmer 1961, Corday and Irving 1961).

Both sinus arrhythmia and sinus arrest can be seen in healthy individuals, but they can also be caused by various external stimuli, for example by anaesthesia and surgery (Dodd *et al.* 1962) or by increased intracranial tension (Corday and Irving 1961).

Young *et al.* (1951), working on dogs anaesthetized with pentobarbital, demonstrated that hypercapnia greatly increased the effect of vagal stimulation on the heart

Downing and Siegel (1963) showed that the sympathetic electrical discharge to the heart was phasically inhibited with inflation of the lungs Hypoxia or hypercapnia increased both sympathetic and parasympathetic discharge to the heart resulting in bradycardia This bradycardia could be abolished by atropine indicating an over whelming vagal effect on the S A node

Sechzer *et al* (1960) were able to demonstrate an accentuation of sinus arrhythmia in healthy male volunteers during inhalation of 7 to 14 per cent carbon dioxide in oxygen

In cardiac rhythm hypercapnia seems seldom to cause severe irregularities in man or in dog A common finding during hypercapnia is an increased effect of vagal stimulation on the heart

C Effects of anaesthesia, relaxants and thoracotomy

Much attention has been paid to the effects of anaesthesia during haemodynamic studies The wide variety of anaesthetic agents used in physiological experiments is certainly partly responsible for the variable and confusing results (Shabetai *et al* 1963 Barlow and Knott 1964)

Fundamental cardiovascular reactions seem to be depressed or abolished during anaesthesia (Sarajas 1961) Rushmer (1961) stated that in many experiments low reactivity to CO_2 breathing is apparently due to depression by anaesthetic agents and cardioregulatory influences from the central nervous system are impaired by anaesthesia Respiratory acidosis did not cause any variations in mean arterial blood pressure heart rate and total peripheral resistance during cyclopropane (Etsten 1957) or nitrous oxide halothane anaesthesia (Auld *et al* 1962) Deep anaesthesia has purposely been used to prevent cardiac arrhythmias or arrest during hypothermia (Drew and Anderson 1959)

The action of various barbiturates differs considerably Pentobarbital is known to have anticholinergic effects Its use suppresses sinus arrhythmia and causes tachy cardia (Sarajas 1961 Shabetai *et al* 1963) Barlow and Knott (1964) observed that mean arterial pressure heart rate cardiac output and peripheral resistance were markedly increased in dogs anaesthetized with pentobarbital (Nembutal)

The effects of thiopental on cardiovascular dynamics are not so clear and profound Myocardial depression in a dog heart lung preparation was less marked with anaesthetic concentrations of thiopental than with ether cyclopropane or nitrous oxide (Price and Helrich 1955) The sympathetic nervous activity was not increased conspicuously by thiopental (Price 1960 b) This was confirmed by Etsten and Li (1960) when they demonstrated that the effect of thiopental on sympathetic nervous activity was very small as compared with that of ether and cyclopropane In their study the catecholamine concentration in the plasma was not increased during thiopental anaestesia

Studies concerning cardiac output during thiopental anesthesia have given variable results (Dwyer and George 1960) However when thiopental was used alone instead of in combination with other agents there was no consistent change (Price 1960 b) A rapid injection of thiopental (22 5 mg/ kg in less than 5 sec) reduced the myocardial contractile force by nearly 50 per cent but if the injection time was 40—60 seconds the reduction was less than 10 per cent (Brown and Mowlem 1960) Central venous

pressure was not increased during thiopental anaesthesia (Price and Helrich 1955) and right atrial pressure was either unchanged or increased (Price 1960 b)

The effect of succinylcholine on dogs differs markedly from the effects it exerts on man (Purpura and Grundfest 1956 Bullough 1959 Conway 1961 Galindo and Davis 1962 Graf *et al* 1963) In dogs moderate doses of succinylcholine have no effect on blood pressure or heart rate (Bovet *et al* 1951—52) and its effect on the central nervous system and autonomous system is very slight (Bovet and Bovet Nitti 1955) Holmdahl (1956) observed that succinylcholine in a dose of 1 mg/kg during artificial ventilation never caused any changes in systemic blood pressure or in heart rate Price (1960 b) also concluded that its action on sympathetic nervous activity was very weak

Thoracotomy changes intrathoracic balance and modifies cardiac function Rushmer *et al* (1954) observed that the area of the cardiac silhouette and the size of the left ventricle diminished after thoracotomy Fermoso *et al* (1964) observed that thoracotomy increased the extracardiac pressure and caused a reduction of 18 8 per cent in cardiac output Similarly Li *et al* (1960) observed a reduced cardiac output and increased total peripheral resistance following opening of the thorax in anaesthetized man

III OBJECT OF THE INVESTIGATION

In clinical anaesthesia and surgery the normal respiratory pattern is disturbed and often replaced by artificial ventilation This abolishes the body s controlling mechanism and in spite of every effort to keep the carbon dioxide tension within normal limits either hypercapnia or hypocapnia may occur This initiates cardiovascular responses and may disturb the cardiovascular homeostasis although during anaesthesia and surgery the cardiovascular reactions caused by changes in pCO may be masked by other simultaneous phenomena

The aim of the present work was to investigate under very light anaesthesia the effects on cardiovascular haemodynamics of varying arterial carbon dioxide tension In order to elucidate these questions the following data were recorded under low normal and high arterial carbon dioxide tension

1 Heart rate
2 Mean arterial blood pressure
3 Cardiac output
4 Right atrial pressure
5 Cardiac rhythm, with special reference to sinus arrhythmia

On the basis of these data the calculations were performed for

6 Stroke volume
7 Total peripheral resistance

IV MATERIAL AND METHODS

A Material

All experiments were performed on healthy mongrel dogs The dogs had been in the laboratory colony for at least one week under the inspection of veterinary personnel All animals having any sign of illness were discarded and the selected animals were taken from the healthy colony The precise age of the dogs was not known but all animals were full grown dogs Altogether 41 animals males and females with body weights varying between 9 2 kg and 25 kg were used Of these 41 test animals 6 were discarded because the animals either died or deteriorated during the experiment or because the results were otherwise unreliable owing to technical or surgical inaccuracies Thus the experimental results are based on 35 dogs

B Anaesthesia

No premedication was given to any of the animals prior to the experiment Anaesthesia was induced with thiopental sodium (Pentothal Abbott) 20 mg per kg of body weight intravenously The dogs were then intubated with a cuffed endotracheal tube to facilitate the removal of saliva and mucus from the respiratory tract and to permit artificial respiration None of the dogs was tracheotomized Apnoea was induced by giving succinylcholine (Anectine Burroughs Wellcome & Co) 6 – 12 mg intravenously every 20 – 30 min

After endotracheal intubation artificial ventilation was started via the endotracheal tube with 100 per cent oxygen by means of a Bird respirator (combination of Marks 4 and 8) with 200 cc/kg/minute at a frequency of 10 – 12/min Apnoea and ventilation were kept constant during the surgical preparation of the animal The idea was to adjust the artificial ventilation to correspond approximately to that of the animal s normal breathing

C General procedures

1 Surgical procedures

A surgical preparation was performed after anaesthetizing the animal and connecting it to the respirator The right femoral artery and vein were exposed and a polyethylene catheter was placed in the artery for pressure and blood gas determination Another polyethylene catheter was inserted into the femoral vein for drug injections and pressure determinations The tip of the catheter was placed in the right auricle and the position confirmed by means of pressure measurements Both catheters were filled with a heparin – 5 dextrose solution 0 08 mg heparin in one cc of solution

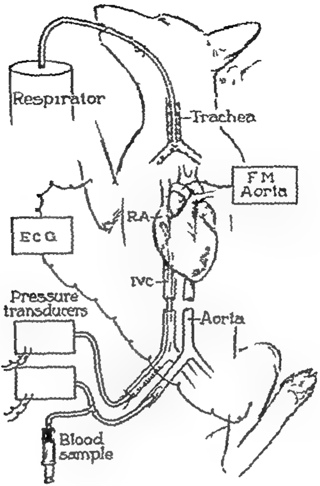

Fig. 1 Scheme of the experimental preparation

A right lateral thoracotomy was performed through the fourth intercostal space. The pericardium was opened medial to the phrenic nerve. The ascending aorta was dissected free from the surrounding tissue and a silk was placed around the root of the aorta. The right atrium was gently pushed downwards and with the silk as a guide an electro-magnetic probe of the proper size was placed around the ascending aorta. The preparation used in the experiments is illustrated in Fig. 1.

The blood loss caused by surgery was carefully replaced with 1–2 day old citrated dog's blood which was kept in plastic bags (Fenval) and stored in a refrigerator.

2. Experimental procedure

The experiment was started by giving 100 per cent oxygen under controlled ventilation. In order to avoid any changes in circulation or arterial pCO_2 due to respiration, the ventilation was kept constant throughout every experiment. The control values of all parameters were recorded and the first arterial blood sample was taken simultaneously. The ventilation was then changed to a mixture of CO_2 (5%) and O_2 (95%) (O_2 and CO_2 delivered by Puritan Gas). When the pCO_2 values seemed to show no more marked changes, a second sample

was taken The time between the first and second samples varied in different animals from 7 to 16 minutes As the second sample was analyzed the ventilatory gas was changed to 100 per cent oxygen again As the pCO_2 values seemed to have reached a constant level again the third blood sample was taken The time after the start of 100 per cent O_2 ventilation varied from 8 to 20 minutes The moments when the samples were taken were always marked on the continuously running recording paper The experiment was concluded when the third sample had been taken

3 *Blood sampling*

Blood samples were drawn from the femoral artery through the polyethylene catheter for gas analyses and pH determinations Luer Loc glass syringes were used previously heparinized with a heparin 5% dextrose solution 5 mg heparin in one cc of 5 per cent dextrose solution The catheters were flushed with a solution of 0 08 mg heparin in one cc of 5 per cent dextrose (Abbott) Before the sample was taken the solution in the catheter was drawn into another syringe with the first portion of blood and discarded Air was carefully excluded 3—4 ml blood for samples was then taken and analyses were performed immediately using the apparatus placed in the same room

D Measuring and recording techniques

1 *Systemic arterial and right atrial pressures*

Arterial blood pressure was measured by connecting the arterial polyethylene catheter to a Statham pressure transducer No P23AA or P23Dc For right atrial pressure measurement the transducer was Statham P23BB The transducers were calibrated with standart mercury or water manometers before each experiment For amplification and recording a Sanborn 150 recording system was used The transducers were connected to a Sanborn 1500—1100 carrier preamplifier and the signals given by the amplifier continuously recorded using a Sanborn recorder Model 158—100B The mean arterial pressure was obtained by means of an electronic intergrator placed in the arterial pressure amplifier

2 *Electrocardiogram (ECG)*

In most experiments the standard ECG lead II was recorded continuously with hypothermic needles as electrodes The preamplifier was a Sanborn model 150—1600 and the recording system the same as previously described For ECG waveform records a paper speed of 25 mm/sec was used

3 *Heart rate (HR)*

Heart rate was counted from the recorded ECG or by observing ECG on a special oscilloscope In an experiment where the ECG was not taken the heart rate was counted from the arterial blood pressure recording All recordings were taken during several respiratory cycles in order to avoid errors due to respiratory arrhythmia

4 *Cardiac output*

The blood flow in the ascending aorta was taken as a measure of the cardiac output (actually total cardiac output minus coronary artery blood flow) This blood flow was

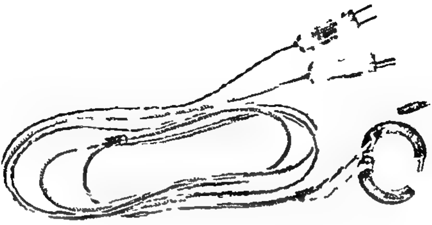

Fig 2 Electromagnetic probe

EMP lead

Aorta

EMP

RA

Fig 3 Electromagnetic probe attached around the root of the aorta EMP lead = lead connecting electromagnetic probe to the flowmeter EMP = Electromagnetic probe RA = right atrium

continuously measured with an electromagnetic flowmeter (EMF). A sinewave EMF Model K 2000 Microflow (Medicon a division of Quality Precision Products Inc Los Angeles Calif) was used. The electromagnetic probe (EMP) (Flowprobe sensors K type) (Fig 2) was attached around the root of the ascending aorta (Fig 3). Probes with a lumen diameter of 12 to 14 mm were used.

The size of the probe was always selected to make a tight connection around the root of the ascending aorta throughout the whole cardiac cycle but not to diminish the lumen of the aorta. This made possible an exact blood flow reading with the smallest shift in zero line. The EMF was connected to a Sanborn high gain preamplifier model 150–2700 and the amplified signal was recorded by the same system as the blood pressure and ECG (Sandborn model 158–100B). The calibration of EMF was made by recording the pulsatile flow and using the reading during the latter part of diastole as a zero line (Barnett and Jacson 1963) because the blood flow in the ascending aorta is practically zero during the latter part of diastole. Changes in instantaneous blood flow could easily be recorded since EMF had a flat response up to a frequency of 100 cycles per second (Ferguson and Wells 1959, Barnett and Jacson 1963).

The calibration of EMF was done several times during each experiment. By frequent calibration of the instrument the shifting in zero line and error in accuracy were eliminated. The mean blood flow was monitored by using electronic damping, which gave only slight pulsations on the recorded curve.

In order to facilitate comparison of the results observed in different experiments and to avoid a possible error in the calibration of various flow probes the cardiac output was recorded in relative »flow units» instead of in absolute values of cc min. The calibration of EMF and changes in readings are linearly related to the flow rate and this makes readings in »flow units» between different experiments directly comparable. On the other hand variations in cardiac output may be compared only as percentages, because of the differences in the size of the experimental animals.

5 *Stroke volume*

Stroke volume was obtained by dividing the systemic blood flow (cardiac output) per minute by the heart rate. Cardiac output was determined in flow units and therefore the calculated stroke volume is also expressed in relative units instead of cc. These units are linearly proportional to each other within each experiment in per cents as well as if measured in cc.

6 *Total peripheral resistance (TPR)*

Total peripheral resistance was calculated as the ratio of mean arterial blood pressure in millimetres of mercury to systemic blood flow (cardiac output) in units per minute (Marshall *et al* 1962).

Since the cardiac output was calculated in flow units the values of peripheral resistance were calculated correspondingly. These are linearly related to the absolute values and so within each experiment are comparable with each other.

7 *Measurement of pH, pCO_2 and pO_2 in arterial blood samples*

For the determinations of pH, pCO_2 and pO_2, 3–4 cc of arterial blood was taken, and the measurement was made by means of an Instrumentation Laboratory model 105 system.

The pH-electrode was a glass electrode (No 107—1) connected with a reference electro (No 107—2) both installed in a water bath The temperature of the bath was controll with a thermometer and a thermistor was used as a stable temperature sensing elemer providing a constant temperature environment (±0 03 C) for the electrode system T temperature was kept at 38 C throughout the experiment

The pCO_2 electrode was a Severinghaus glass electrode installed in the water ba The pO_2 electrode was a Clark electrode installed in the same water bath

Every electrode was calibrated before every experiment For the pH electrode a speci 1L pH solution was used The pCO_2 electrode was calibrated with a special 5 per cent CO_2 gas mixture The pO_2 electrode was calibrated with 100 per cent N_2 gas for zero and room air for another reading Here the partial pressure of oxygen was calculated in mmHg as 20 per cent of the momentary barometric pressure The calibration of the pO_2 electrode was checked with room air before or after every measurement

The actual study is based on changes in arterial carbon dioxide tension The measurements of pH and pO_2 were done only to check the animal s condition and in order to avoid hypoxia

E Statistical analysis

The standard deviation of the means in mean arterial pressure heart rate and right atrial pressure were determined by the usual formula The statistics obtained in this manner have been given in connection with the respective results

The correlation between changes in arterial carbon dioxide tension and changes in heart rate mean arterial pressure right atrial pressure and standard deviation of sinus arrhythmia was determined by means of single regression analysis The significance of the correlations between these variables was also determined by means of a sign test Changes in total peripheral resistance cardiac output and stroke volume caused by variations in pCO_2 were likewise determined by means of a sign test In addition the correlation between the simultaneous changes in heart rate and stroke volume was also determined

The statistical significance of the coefficient of correlation (r) coefficient of regression (b) and sign test was graded as »almost significant« (* = $p < 0.05$) »significant« (** = $p < 0.01$) and »highly significant« (*** = $p < 0.001$)

V RESULTS

The experiments are divided into groups according to the type of change in arterial carbon dioxide tension The main division is made according to the direction of CO_2 change in one main part the observations were made during increasing pCO_2 in the other during decreasing pCO_2 Each main part is divided into three sub-groups according to the level of pCO_2 i e normo-hypercapnia hypo-normocapnia and hypo-hypercapnia and vice versa Normocapnia includes pCO_2 values between 35 mmHg and 45 mmHg and hypocapnia values below 35 mmHg while values above 45 mmHg are classed as hypercapnia

The response of various cardiovascular functions to changes in arterial pCO_2 was determined in both main parts by observing differences in every parameter in the sub-groups Thus observations were made during changes in pCO_2 from normocapnia to hypercapnia and vice versa, and similar observations were made between hypo- and normocapnia and hypo-hypercapnia For some functions this comparison has only been made qualitatively while others have been examined in greater detail

1 *Heart rate (HR)*

Observations on the heart rate were made in 118 experiments performed on 33 dogs and a comparison of changes in HR was made in 64 cases The results are shown in Tables 1 2 and 11 and in Figs 4 8 and 12 The mean heart rate during hypocapnia was 177 ± 37/min in normocapnia it was 155 ± 34/min and in hypercapnia 134 ± 26/min

The reduction of heart rate in the experiments involving increasing pCO_2 is consistent in all observations throughout this main part The reverse can be seen in decreasing pCO_2 in all but three experiments (dogs no 28 32 33) Here the change

Table 1 Heart rate and changes in pCO_2

pCO_2	$CO_2\uparrow$ Heart rate				$CO_2\downarrow$ Heart rate			
	+	±	−	p	+	±	−	p
Normo hyper normo	−	−	15	0012	17	4	3	002
Hypo normo hypo	−	−	6	032	5	2	−	062
Hypo hyper hypo	−	−	9	004	3	−	−	412
Total	−	−	30	0000	25	6	3	0003

Table 2 The effect of changes in pCO on heart rate

pCO_2	Heart rate	$CO_2\uparrow$						$CO\downarrow$					
		Heart rate						Heart rate					
		n	+	n	±	n	−	n	+	n	±	n	−
Normo hyper normo	mean	–		–		15	134→124	17	124→162	4	123→122	3	149→147
	range						(104−214)→ (96−174)		(96−190)→ (105−220)		(120−126)→ (1[illegible]0−126)		(1[illegible]0−182)− (118−180)
Hypo normo hypo	mean	–		–		6	196→152	5	152→188	2	210→210	–	–
	range						(155−218)→ (100−180)		(126−210)→ (130−2[illegible]4)		(210−210)→ (210−210)		–
Hypo hyper hypo	mean	–		–		9	172→125	3	120→152	–	–	–	–
	range						(130−224)→ (98−152)		(120−120)→ (140−170)		–		–
Total	mean	–	–	–	–	30	174→134	25	132−167	6	167→166	3	149→147
	range				–		(104−224)− (98−180)		(96−210)→ (105−224)		(1[illegible]0−210)→ (120−210)		(120−182)→ (118−180)

n = number of experiments

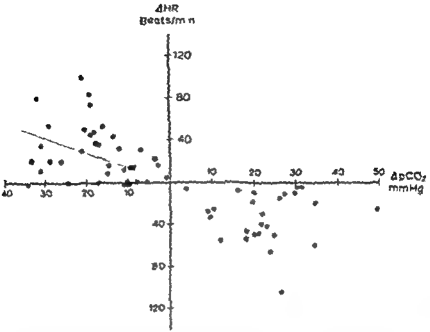

Fig 4 Correlation between changes in arterial pCO and changes in heart rate (HR) Each dot represents a different experiment r=0 078 and ΔHR 0 093 1 400 ΔpCO

from hypercapnia to normocapnia was followed by a decrease of two beats per minute in each of three cases Table 2 shows actual heart rate values under different experimental conditions

The effect of changing pCO_2 on the heart rate is highly significant both in increasing pCO_2 (p = 0000***) and in decreasing pCO_2 (p = 0003***) and similarly in the entire series of experiments The correlation between changes in pCO_2 and changes in heart rate can be seen in Table 11 and in Fig 4 The correlation coefficient is r −0 758 and the regression equation ΔHR = 0 083−1 400 ΔpCO_2

2 Mean arterial pressure (ABP)

This part of the study consists of 111 experiments performed on 32 dogs A comparison of changes in mean arterial pressure was made in 68 cases The results can be seen in Tables 3 4 and 11 and in Figs 5 6 7 and 8 The average mean arterial

Table 3 Mean arterial pressure and changes in pCO

pCO	CO				CO_{2}			
	Arterial pressure				Arterial pressure			
	−	+		p	−	+	−	p
Normo hyper normo	6	5	4	754	5	6	17	016
Hypo normo hypo	[illegible]	1		008		3	4	_59
Hypo hyper hypo	8		1	124		1	1	798
Total	20	6	5	004	5	10	22	007

Table 4 The effect of changes in pCO_2 on mean arterial pressure

pCO_2	Arterial pressure	CO ↑						CO_2 ↓					
		Arterial pressure						Arterial pressure					
		n	+	n	±	n	−	n	+	n	±	n	−
Normo hyper normo	mean range	6	130→147 (110–150)→ (130–170)	5	134→135 (100–155)→ (100–155)	4	149→134 (136–190) (100–170)	5	130→140 (100–150)→ (110–160)	6	125→125 (100–170) (102–170)	17	139→125 (98–190)→ (95–150)
Hypo normo hypo	mean range	8	123→139 (100–150) (110–160)	1	125→125	—	– –	—		3	128→1.9 (124–136) (125–136)	4	133→122 (1.0–155)→ (100–150)
Hypo hyper hypo	mean range	6	121→141 (96–157)→ (110–180)	—	–	1	150→140	—	– –	1	130→130	1	170→145
Total	mean range	.0	125→142 (96–152)→ (110–190)	6	129→130 (100–155)→ (100–155)	5	149→137 (136–180)→ (100–170)	5	130→140 (100–150)→ (110–160)	10	128→128 (100–170)→ (102–170)	2.	147→131 (98–190)→ (95–150)

n = number of experiments

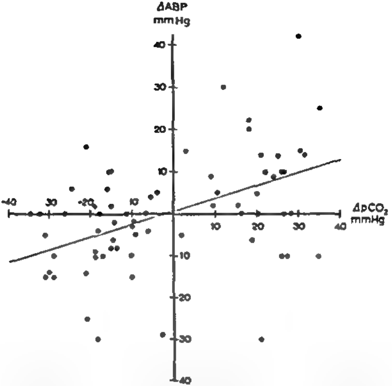

Fig 5 Correlation between changes in arterial pCO_2 and changes in mean arterial pressure (ABP) Each dot represents a different experiment $r = 0\,462$ and $\Delta ABP = 0\,859 + 0\,302\ \Delta pCO$

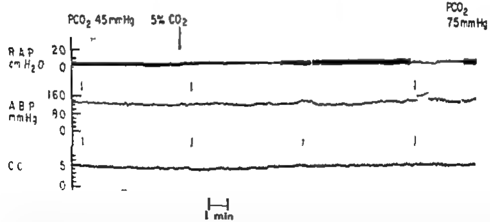

Fig 6 Dog no 23 The effect of increasing pCO_2 on right atrial pressure (RAP) mean arterial pressure (ABP) and cardiac output (CO) Note the stability of ABP before 5 % CO_2 was started and the increasing fluctuation of ABP when pCO_2 rises The immediate fall in CO after the start of 5 % CO_2 and the later increase in cardiac output (CO) can also be seen

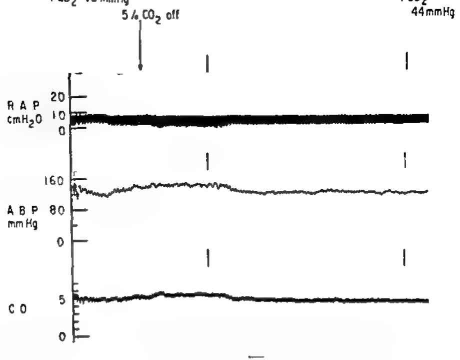

Fig 7 Dog no 23 Effect of decreasing pCO_2 on right atrial pressure (RAP) mean arterial pressure (ABP) and cardiac output (CO) Note the fluctuation of ABP during high pCO_2 and overshooting of ABP after cessation of 5 CO_2 later followed by more stable phase Similar phenomena can also be seen in the cardiac output recording

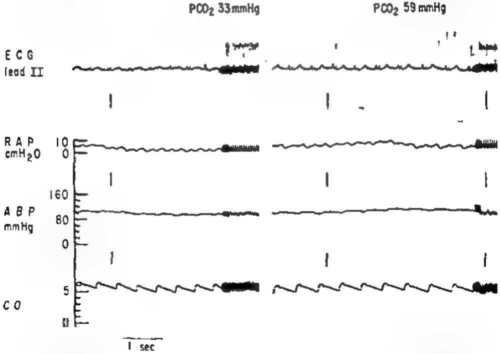

Fig 8 Dog no 15 Effect of different levels of arterial pCO_2 on heart rate right atrial pressure (RAP) arterial blood pressure (ABP) and cardiac output (CO)

pressure was 127 ± [illegible] mmHg (range 96–152 mmHg) in hypocapnia 133 ± 20 mmHg (95–180 mmHg) in normocapnia and 132 ± 20 mmHg (98–180) in hypercapnia

In increasing pCO_2 the rise in pCO_2 elevates the mean arterial pressure significantly although variations can be seen in different sub-groups When pCO_2 is decreased similar variety can be seen in changes of arterial blood pressure in the smaller groups *but as a whole the correlation between decreasing pCO_2 and decrease in blood pressure* [illegible] significant

The correlation between changes in arterial pCO_2 and mean arterial pressure [illegible] the entire series of experiments can be seen in Table 11 and in Fig 5 The correlation coefficient is $r = 0\,462$ and the regression equation $\Delta ABP = 0\,859 + [illegible]\,302\,\Delta pCO_2$ Both these values indicate a highly significant correlation

In some of the experiments the arterial blood pressure showed a particular pattern Sometimes immediately after the change in the concentration of the inspired gas mixture an overshooting of the blood pressure or reversal of direction was seen as compared with the blood pressure values observed later under balanced conditions (Figs 6 and 7) *In some other experiments on lower and normal pCO_2 values the blood* pressure remained stable at a certain level but during hypercapnia a fluctuation of 10–30 mmHg could be observed on several occasions This fluctuation disappeared again when pCO_2 was lowered to or below the normal range (Figs 6 and 7)

3 Total peripheral resistance (TPR)

The total peripheral resistance was calculated in 89 experiments performed on 23 dogs and a comparison of changes in TPR was made in 56 cases The evaluation of the findings is only qualitative and the results can be seen in Table 5

Table 5 Total peripheral resistance and changes in pCO_2

pCO_2	$CO_2\uparrow$ Peripheral resistance				$CO_2\downarrow$ Peripheral resistance			
	+	±	−	p	+	±	−	p
Normo hyper normo	7	3	—	016	5	[illegible]	11	210
Hypo normo hypo	4	1	2	688	3	—	3	~1 0
Hypo hyper hypo	[illegible]	—	1	040	—	—	2	625
Total	19	4	3	0018	[illegible]	6	16	152

In changes of total peripheral resistance a difference is seen between the groups of increasing pCO_2 and decreasing pCO_2 Increasing pCO_2 elevates total peripheral resistance and this trend [illegible] seen especially with higher pCO_2 values of this group Decreasing pCO on the contrary does not have a consistent effect on the TPR and the changes caused by decreasing pCO_2 are quite variable This is seen at all pCO_2 levels

When all experiments are examined together the difference between high and low pCO_2 [illegible] obvious High pCO tensions seem to have marked effect on TPR while the correlation between pCO_2 and TPR in hypo- and normocapnia is questionable

Increasing pCO_2 elevates TPR in 73 per cent of 26 experiments The result is statistically significant ($p = 0018^{**}$) During decreasing pCO TPR was lowered only

in 53 per cent of 30 experiments which indicates that there is no significant correlation between these two variables (p = 152)

4 *Cardiac output*

The cardiac output was recorded in 89 experiments performed on 23 dogs A comparison of changes in cardiac output was made in 56 cases The results can be seen in Table 6 and in Figs 6 7 8 and 9

It can be seen that changes in cardiac output do not follow changes in carbon dioxide tension During both increasing and decreasing pCO_2 the change in cardiac output showed an almost equal dispersion in both directions (Fig 9) Statistical analysis shows no correlation between these two parameters

Table 6 Cardiac output and changes in pCO_2

pCO_2	$CO_2\uparrow$				$CO_2\downarrow$			
	Cardiac output				Cardiac output			
	+	±	−	p	+	±	−	p
Normo hyper normo	2	2	6	290	8	2	12	504
Hypo normo hypo	4	2	1	376	2	—	4	688
Hypo hyper hypo	3	2	4	~1 0	2	—	—	625
Total	9	6	11	824	12	2	16	678

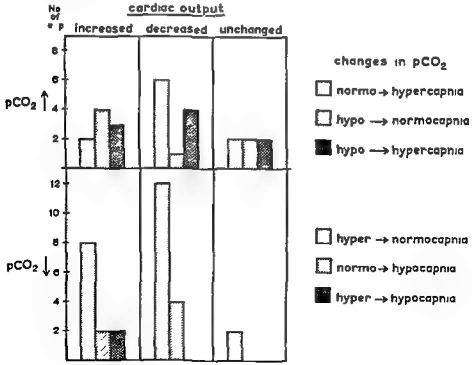

Fig 9 Effect of changing pCO_2 on cardiac output

The initial change in cardiac output after changing the repiratory gas mixture often differed from later findings In several experiments the direction of the initial change was greater or opposite to a more stable reading observed later This phenomenon was perceptible both after starting 5 per cent CO_2 (Fig 6) and after changing the gas mixture to 100 per cent oxygen (Fig 7) This phenomenon when it occurred lasted from 2 to 5 minutes The fluctuation of the cardiac output was also observed on several occasions during high CO_2 and disappeared when CO_2 tension was reduced back to normal values (Fig 7) This fluctuation seemed to be connected with respira tory sinus arrhythmia

5 *Stroke volume*

Stroke volume was calculated in 80 experiments performed on 21 dogs and a comparison of changes in stroke volume was made in 50 cases The comparison of the findings was only qualitative and the results can be seen in Table 7

Table 7 Stroke volume and changes in pCO_2

pCO_2	$CO_2\uparrow$				$CO_2\downarrow$			
	Stroke volume				Stroke volume			
	+	±	−	p	+	±	−	p
Normo hyper normo	6	1	2	290	3	—	16	004
Hypo-normo hypo	6	—	—	032	—	—	5	06.
Hypo hyper hypo	8	1	—	008	—	—	2	625
Total	20	2	2	0011 *	3	—	23	0008

In both main parts of the experiments the findings are quite evident Increasing pCO_2 caused a consistent rise in stroke volume ($p = 0011$**) which [redacted] also seen in all sub-groups In decreasing pCO_2 the findings are similar ($p = 0008$***) In most experiments changes in stroke volume followed a decrease in arterial pCO_2

When variations in stroke volume caused by changes in arterial pCO_2 were com pared with corresponding variations in heart rate the correlation was quite evident In the entire series ($n = 80$) the correlation is highly significant ($r = 543$) (Table 12)

6 *Right atrial pressure (RAP)*

Right atrial pressure was recorded in 90 experiments performed on 28 dogs and a comparison of changes in RAP was made in 53 cases The results of the experiments

Table 8 Right atrial pressure and changes in pCO_2

pCO_2	CO $\uparrow$				$CO_2\downarrow$			
	Right atrial pressure				Right atrial pressure			
	+	±	−	p	+	±	−	p
Normo hyper normo	11	1	—	009	—	3	20	0001
Hypo normo hypo	[redacted]	1	—	625	—	—	4	259
Hypo-hyper hypo	8	1	—	008	—	—	[redacted]	625
Total	21	3	—	0001**	—	3	26	0000**

Table 9 The effect of changes in pCO_2 on right atrial pressure

pCO_2	RAP	CO_2 ↑						CO_2 ↓					
		Right atrial pressure						Right atrial pressure					
		n	+	n	±	n	−	n	+	n	±	n	−
Normo hyper normo	mean	11	3 4 7 0	1	0 →0	—	—	—	—	3	3 3– 3 3	.0	6 3 →3 6
	range		(0–8)→ (2–12)						—		(−1– +9) (−1– +9)		(2–12) → (0–9)
Hypo normo hypo	mean	?	3 0→5 5	1	4 →4	—	—	—	—	—		4	3 8 →2 0
	range		(1– +7) → (1–10)						—				(−2–+10) (−4–+7)
Hypo hyper hypo	mean	8	4 3→6 3	1	−1 →−1	—	—	—	—	—	—	2	8→6
	range		(0–10)→ (1–12)				—		—		—		(4–12)→ (2–10)
Total	mean	21	3 6→6 3	3	1 →1	—	—	—	—	3	3 3– 3 3	26	6 0– 3 9
	range		(−1– +10)→ (1–12)		(−1– +4)→ (−1– +4)		—		—		(−1– +9)→ (−1– +9)		(−2– +12)→ (−4– +10)

n = number of experiments

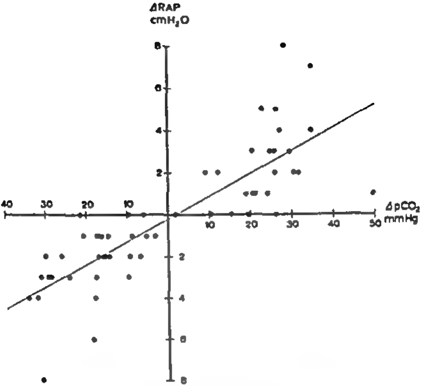

Fig 10 Correlation between changes in arterial pCO_2 and changes in right atrial pressure (RAP) Each dot represents a different experiment r = 0 8 6 and 1 RAP = −0 231 + 0 109 ΔpCO_2

are shown in Tables 8 9 and 11 and in Figs 6 7 8 and 10 The mean right atrial pressure was 3 3 ± 3 6 cm H_2O (from −4 to + 10 cm H O) during hypocapnia 3 2 ± 2 8 cm H_2O (−2− + 10 cm H_2O) during normocapnia and 4 7 ± 3 7 cm H_2O (−1− + 12 cm H_2O) in hypercapnia

The direct relationship between the changes in arterial pCO_2 and right atrial pressure was unquestionable throughout the study With increasing pCO_2 right atrial pressure mostly followed changes in pCO_2 (Figs ▮and 8) In 3 out of 24 experiments no change could be seen and changes in the reverse direction were never observed *Similarly decreasing pCO caused a reduction in right atrial pressure in* all but 3 out of 29 experiments (Fig 7) In no instance was a decreasing pCO_2 followed by an increase in right atrial pressure Previously described changes could be seen in all three sub-groups of both main parts not only in hypercapnia but also in normo- and hypocapnia

However there were differences according to different tensions of arterial CO_2 The mean RAP in hypocapnia (3 3 ± 3 6 cm H_2O) and normocapnia (3 2 = 2 8) did not differ much but the change from normocapnia to hyperpapnia caused a marked increase in RAP (from 3 2 ± 2 8 cm H_2O to 4 7 ± 3 7 cm H_2O) In most individual experiments however the difference in RAP between various pCO_2 groups was more marked

Table 11 Effect of arterial pCO_2 changes on variations in heart rate (HR) mean arterial pressure (ABP) right atrial pressure (RAP) and sinus arrhythmia ($SD_{R\ R}$)

Number of observations	Dependent variable	Constant	Regression coefficient	S E of regression coefficient	Test variable	Correlation coefficient
64	ΔHR	0 083	−1 400	0 147	9 52* *	0 758
68	ΔABP	0 859	+0 302	0 071	4 25***	0 462
53	ΔRAP	−0 231	+0 109	0 010	10 90 *	0 826
43	$\Delta SD_{R\ R}$	−0 035	+0 045	0 005	9 18***	0 814

Table 12 Correlation between different variables during changes of pCO_2 The upper number is the correlation coefficient and the lower number (in parentheses) indicates the number of observations

	Stroke volume	ABP	RAP	TPR
HR	− 543*** (80)	012 (99)	247* (78)	− 228* (80)
Stroke volume		− 214 (80)	100 (58)	− 532** (80)
ABP			100 (85)	511** (89)
RAP				255 (66)

The effects of arterial pCO_2 changes on variations in heart rate mean arterial pressure right atrial pressure and sinus arrhythmia are collected in Table 11 Correlations between different cardiovascular parameters during arterial pCO_2 changes are shown in Table 12

VI DISCUSSION

Changes in arterial carbon dioxide tension lead to responses in several organ systems. In the cardiovascular system changes in pCO_2 elicit processes influencing most of the cardiovascular parameters

Cardiac output is the basic measure of blood supplied to all parts of the body In adjusting the cardiac output several factors must be taken into consideration The two main factors are heart rate and stroke volume The stroke volume depends on the amount of blood the heart can pump into the arteries against the peripheral resistance Right atrial pressure intrathoracic pressure and arterial pressure in conjunction with total peripheral resistance and contractility of the heart muscle take part in adjusting the stroke volume (Rushmer 1961) All these parameters are affected by carbon dioxide and so is the cardiac output In addition to this thoracotomy changes intrathoracic pressure and also the heart s ability to pump blood (Rushmer *et al* 1954 Li *et al* 1960)

Most studies on the effect of carbon dioxide on the *heart rate* in dogs have shown bradycardia in response to hypercapnia (Brown and Miller 1952 b Boniface and Brown 1953 Clowes *et al* 1955 Holmdahl 1956 Feinberg *et al* 1960 Manley *et al* 1964) but tachycardia has also been reported (Stone *et al* 1958 Downing *et al* 1963) Spontaneous breathing during exposure to CO_2 considerably increases the respiratory effort resulting in an increase in heart rate Experiments under relaxants and with controlled respiration have shown bradycardia in response to hypercapnia

In the present study the heart rate always decreased with increasing carbon dioxide tension both during a change from hypocapnia to normocapnia and when pCO_2 was raised to higher values The opposite phenomenon was seen in most cases when the pCO_2 was lowered This was also seen when the carbon dioxide tension was lowered to subnormal values Even in the hypocapnic state lowering of pCO_2 increased the heart rate This seems to indicate not only that hypercapnia causes bradycardia but that chronotropic effect of CO_2 remains equally intense at a subnormal arterial carbon dioxide tension In contrast to active hyperventilation the present study was made under controlled respiration where the respiratory pattern remains constant irrespective of CO_2 tension and does not effect the heart rate

It has been shown that bradycardia also occurs in dogs during hypercapnia after division of the vagi (Nahas and Cavert 1957) In view of previous findings it is obvious that in spite of a vagal effect CO_2 has a direct effect on the rate regulating mechanism Whether carbon dioxide exerts a direct effect or acts by changing the hydrogen ion concentration cannot be concluded from the present study

Hypercapnia of the separated central nervous system causes tachycardia in dogs (Downing *et al* 1963) in contrast to the slowing effect of generalized hypercapnia

observed here This phenomenon indicates that in spite of sympathetic stimulation which primarily affects most of the parameters followed in the present study the vagal and local effects of increased pCO_2 on HR override the sympathetic action and lead to a decrease in heart rate The present study supports the view that in a state where the carbon dioxide tension is equal throughout the body the heart rate is inversely proportional to the arterial carbon dioxide tension whether there is normo hyper or hypocapnia

In addition to variations in heart rate irregularities in *cardiac rhythm* can be seen during hypercapnia although they are not very common The high carbon dioxide tolerance of the human and dog heart has frequently been demonstrated (Holmdahl 1956 McArdle 1959 Sechzer *et al* 1960 Price 1960 a Manley *et al* 1964) Auricular ventricular and nodal extrasystoles are arrhythmias infrequently observed (Brown and Miller 1952 b Miller *et al* 1952 Price 1960 a Manley *et al* 1964) Severe arrhythmias including ventricular fibrillation and death have been reported especially during the posthypercapnic period when CO_2 is rapidly eliminated However these arrhythmias occurred after hour long ventilation with 20—30 per cent carbon dioxide which in clinical practice is hardly ever seen (Itami 1912—13 Miller *et al* 1952 Brown and Miller 1952 a Heath and Brown 1956)

In the present study the results concerning changes in heart rhythm reveal increased vagal activity during hypercapnia The disorder most commonly observed during the experiments was sinus arrhythmia Sinus arrest occurred in six experiments and nodal rhythm only in one ECG was followed at every step of the experiment so that hardly any of the arrhythmias could be missed and no other kinds of arrhythmias were ever observed Thus the results presented in this study give a good overall picture of the abnormalities in heart rhythm during moderate changes in pCO_2 on dogs under thoracotomy

In the present study an increased intensity of sinus arrhythmia nearly always followed an increase in arterial carbon dioxide tension. Sinus arrhythmia was diminished by lowering of pCO_2 and with low pCO_2 tension it could very seldom be seen These results indicate a ri e in vagal activity to the S A node with increasing arterial carbon dioxide tension This is in accordance with a few other reports con cerning increased vagal activity during hypercapnia (Young *et al* 1951 Sechzer *et al* 1960 Downing and Siegel 1963) The effects of sinus arrhythmia and changes in heart rate will be discussed later in this chapter

In the present study changes in *stroke volume* were also closely related to pCO_2 but the findings were opposite to those relating to heart rate Hypercapnia increased stroke volume in most cases and correspondingly stroke volume became smaller with decreasing CO_2 tension This phenomenon could be seen throughout the study both between normo and hypercapnia and between normo and hypocapnia The finding is in accordance with earlier reports concerning CO_2 and stroke volume (Burnum *et al* 1954 Richardson *et al* 1961)

Several investigators have pointed out a progressive increase in stroke volume as heart rate decreases Although heart rate and stroke volume are adjusted independently it is evident that stroke volume is higher at slower heart rate values (Tigerstedt 1913 Berglund *et al* 1958 Warner and Toronto 1960 Miller *et al* 1962 b) As the heart rate increases the stroke volume decreases while no marked alteration

in cardiac output can be seen When the heart rate exceeds 150—180 per minute in the dog the cardiac output tends to decrease (Berglund *et al* 1958 Miller *et al* 1962 b) In the present study the same phenomenon was seen in most experiments The correlation between the changes in heart rate and stroke volume was highly significant so that a slow heart rate was accompanied by higher stroke volume while accelerating heart rate diminished stroke volume The changes in cardiac output caused by this phenomenon are variable

In this study the stroke volume was calculated by dividing the cardiac output by the heart rate per minute This method has generally been accepted and used by many investigators (Sarnoff and Berglund 1954 Berglund *et al* 1958 Miller *et al* 1962 b)

Several investigators have pointed out the importance of *right atrial pressure* (*RAP*) in adjusting the circulation of the blood (Bainbridge 1915 Wiggers and Katz 1922, Stead and Warren 1947 Ferguson *et al* 1953 Guyton 1955 Aviado 1962 Guyton 1963 Fermoso *et al* 1964) RAP is said to indicate the ventricle s ability to pump blood the pressure will rise when the ventricle fails to force the blood on (Guyton *et al* 1959) However this weakness of the heart is often compensated by several homeostatic mechanisms so that no decrease in cardiac output can be seen (Nahas and Cavert 1957 Price 1960 a)

Besides the inability of the heart to pump the available blood *i e* cardiac decompensation there may be other causes for elevated RAP Increased pCO_2 stimulates the brain stem and chemoreceptors leading reflexly to vasomotor activity (Korner 1959) This can increase venomotor tone and so result in venoconstriction (Sharpey Schafer 1961) which in turn may increase the venous return to the heart (Folkow 1955) and increase central venous and right atrial pressure

In the present study changes in right atrial pressure constantly accompanied changes in arterial carbon dioxide tension both in normo- and hypercapnia and in hypocapnia A change in pCO_2 was almost invariably followed by a concomitant change in RAP Variations observed in the basic level of RAP were probably due to differences in experimental animals and in a variety of conditions between different experiments However the experimental conditions in a certain experiment on the same animal were the same throughout the test and so did not affect the final results.

Investigations concerning the effect of carbon dioxide on RAP almost uniformly resulted in increased right atrial and central venous pressure during hypercapnia (Schneider and Truesdell 1922 Nahas and Cavert 1957 Seveso 1962) In the heart lung preparation the elevated RAP during hypercapnia was associated with cardiac failure Elevated central venous pressure was similarly observed in intact animals but no heart failure could be demonstrated its prevention was thought to be due to stimulation of the sympatho-adrenal system (Nahas and Cavert 1957) This is in accordance with the present findings which show a significant rise in RAP without a similar reduction in cardiac output

In hyperventilation the effects of pCO_2 depend greatly on experimental conditions Voluntary hyperventilation decreases central venous pressure (Thompson *et al* 1962) whereas passive hyperventilation with closed chest elevates RAP (Rowe *et al* 1962) In the present study where the chest was open elimination of carbon dioxide made the RAP decrease This can be caused by diminished venomotor tone as a result of decreased CO_2 and by augmentation of myocardial force due to elimination of respiratory acidosis

The adjustment of *arterial pressure* is influenced by many different factors and mechanisms the most important of which are total peripheral resistance heart rate and stroke volume However, the pressoreceptor reflexes may be taken as the only regulatory mechanism (Heymans and Neil 1958 Rushmer 1961) Under normal conditions the cardiovascular system reacts to an elevation in blood pressure with reduction in total peripheral resistance and in heart rate Reduction in stroke volume has also been observed (Carlsten *et al* 1958 Rushmer 1961) If a factor such as increased pCO_2 in the present study is able to elevate the systemic blood pressure this is because it has been able to affect the pressoreceptor mechanism It has been shown that locally CO_2 causes vasodilatation (Gaskell 1880—82 Kety and Schmidt 1946 Fleishman *et al* 1957 Etsten 1957 Diji 1959 Price 1960) but centrally CO_2 produces sympathetic stimulation followed by vasoconstriction (Goldstein and DuBois 1927 McArdle 1959 Tenney 1960 Cross and Silver 1962) At the same time increasing CO_2 tension activates chemoreceptors which send impulses also causing vasoconstriction The final result is a rise in total peripheral resistance and usually an increase in systemic arterial blood pressure In addition to the elevation of blood pressure in the present study the elevation of arterial pCO_2 caused a reduction in heart rate and an increase in stroke volume as well as in right atrial pressure No significant changes in cardiac output could be observed while total peripheral resistance was significantly increased These phenomena indicate a sympathetic stimulation causing a rise in total peripheral resistance and in cardiac function which prevents cardiac output from decreasing in spite of a significant decrease in heart rate Similarly in most other reports hypercapnia has resulted in an increase of arterial blood pressure (Goldstein and DuBois 1927 Dripps and Comroe 1947 McArdle 1959 Sechzer *et al* 1960)

The effects of hyperventilation and hypocapnia on arterial pressure are not so consistent In man there are reports indicating elevated blood pressure (Kety and Schmidt 1946) no significant changes (Norlin 1932 Richardson *et al* 1961) or even a fall in blood pressure (Burnum *et al* 1954) In anaesthetized animals hyperventilation and hypocapnia have been reported to cause a fall in blood pressure (Seevers *et al* 1940 Kety and Schmidt 1946) while in unanaesthetized dogs no fall on blood pressure could be seen (Seevers *et al* 1940) The combined results of the present study indicate that increasing carbon dioxide tension tends to raise the blood pressure and vice versa. This was observed in hypo- normo- and hypercapnia In view of these findings it is obvious that CO_2 exerts a regulating influence on arterial blood pressure in all these states *i.e* both in hypocapnia and in normo- and hypercapnia Similarly changes in TPR mostly followed changes in arterial pCO_2 However during low pCO_2 values the effect seems to be much less pronounced The catecholamines probably do not influence the phenomenon since in this study the CO_2 tension was probably not high enough to cause a release of these agents (Tenney 1956 Honig and Tenney 1957)

The *total peripheral resistance* has here been calculated in arbitrary units as a ratio of mean arterial blood pressure to cardiac output The same method of expression was used by Wiggers (1944) Green *et al* (1944) and Green (1948) In the time between the two readings both arterial pressure and cardiac output may have changed which may cause inaccuracies in quantitative measurements The method is suitable however when measurements are made on the intact dog under controlled conditions and

offers some guide to the extent of TPR changes. In the present study comparisons between different TPR readings were made only qualitatively and only between the results of experiments made on the same dog. Hence the results presented here may be assumed to give reliable information about the TPR under different CO_2 tensions.

The *cardiac output* is the sum of the functions of the various cardiovascular components. In the present study changes in arterial pCO_2 did not show any correlation with simultaneous changes in cardiac output.

CO_2 affects the circulation directly, through the sympathetic nervous system and through chemoreceptors. In high CO_2 concentrations catecholamines are also released from the adrenal medulla. Both depressing and stimulating factors act simultaneously and the magnitude of each component may change in response to the intensity of the stimulus.

In the present study heart rate and stroke volume, the two main components adjusting cardiac output, were significantly correlated and inversely proportional to each other during changes of pCO_2. Other components, such as right atrial pressure, mean arterial pressure and total peripheral resistance, although closely related to changes in arterial pCO_2, were not so closely correlated with each other during pCO_2 changes. With this in mind it is easy to understand that changes of arterial pCO_2 had no concomitant effect on cardiac output. The lack of balance between the various circulatory components was especially evident immediately after the change of inspired CO_2 concentration, when in several experiments both cardiac output and arterial blood pressure fluctuated up and down before reaching a new level.

Observations in other investigations indicate a rise in cardiac output during voluntary hyperventilation during hypo-, normo- or hypercapnia in man (Grollman 1930, Asmussen 1943, Price 1960 a, Wade and Bishop 1962) and in dogs (Marshall 1926, Linde *et al.* 1963), while in other reports the effect of CO_2 breathing is stated to be very variable (Fishman *et al.* 1960). The results during controlled ventilation have also been inconsistent. Paulet and Bernard (1961) and Linde *et al.* (1963) demonstrated an increase in cardiac output during CO_2 exposure, but Auld *et al.* (1962) were unable to demonstrate any significant changes. The effects of thoracotomy on the response of cardiac output to CO_2 breathing are also variable (Rahn and Bahnson 1953, Weil *et al.* 1957, Peters 1957, Manfredi and Sieker 1960, Fishman 1961).

In the present study cardiac output was measured by means of an electromagnetic flowmeter. Since its application to experimental and clinical use, this instrument has frequently been employed for this purpose (Schenk *et al.* 1958, Berglund *et al.* 1958, Spencer and Denison 1960, Jochim 1962 a, 1962 b) and allows an instantaneous as well as continuous determination of blood flow. In the present study the cardiac output measurements and comparisons of changes were made for each animal separately. The EMF was zeroed just before each reading. Thus inaccuracies due to the instrument cannot have had any influence on the results observed.

The results presented here have shown that changes in arterial carbon dioxide tension have a marked influence on the circulation. Most trends during decreasing arterial pCO_2 were the reverse of those observed during an increase of pCO_2.

In *increasing arterial* pCO_2 several simultaneous phenomena could be observed. Arterial blood pressure, total peripheral resistance, stroke volume and right atrial pressure were increased, while in the heart rate a marked slowing could be observed. Augmentation of sinus arrhythmia became more prominent with increasing pCO_2.

and especilly in hypercapnia All these findings were consistently connected with increasing pCO_2

The effect of the above changes on cardiac output was variable Stroke volume was seen to increase with rising pCO_2 but simultaneously slowing of the heart rate was observed Increasing total peripheral resistance increased arterial blood pressure but at the same time it worked against the flow *i e* cardiac output The results observed in the present study indicate that during controlled ventilation increasing arterial carbon dioxide tension and specially hypercapnia caused vasoconstriction and an increase in TPR Changes between subnormal and normal arterial carbon dioxide values did not significantly alter the calculated total peripheral resistance Increasing pCO_2 also raised right atrial pressure consistently in the present study which in turn indicates a rise in central venous pressure Although sympathetic stimulation increases the strength of contraction of both atria and ventricles the simultaneous parasympathetic stimulation observed in the present study decreased both the heart rate and the strength of the atrial contractions (Guyton *et al* 1964) This leads to a rise in stroke volume and RAP without increasing cardiac output Hence the elevated RAP found in the present study did not necessarily increase cardiac output in thoracotomized dogs

An especially noteworthy finding was the fluctuation of arterial pressure and cardiac output after an increase of CO_2 concentration in the inspired gas Sometimes the first change was opposite to later findings This phenomenon was much more marked if 10 per cent CO_2 was added in the inspired air (Galindo 1963) The same kind of fluctuation has been reported in several other investigations especially con cerned with arterial pressure (Clowes *et al* 1955 Euler and Liljestrand 1946 Sealy *et al* 1954 Holmdahl 1956 Manley *et al* 1964) Manley also reported a rapid rise in blood pressure back to the control level or above it after termination of CO_2 breathing It has been stated that the initial fall in blood pressure during hypercapnia is the result of local vasodilatation the subsequent rise occurring when central stimulation exceeds the local effect (Euler and Liljestrand 1946) Sympathetic activation was believed to play an important role in returning arterial pressure towards the control level (Euler and Liljestrand 1946 Manley *et al* 1964) Reduction in cardiac output may also lead to a fall in arterial pressure (Holmdahl 1956) In the light of previous reports and the findings observed in the present study it is obvious that alternation of peripheral vasodilatation and constriction plays an important part in the sudden fluctuations of arterial pressure and cardiac output observed in this study A direct vasodilating effect of CO_2 seems to precede the compensating sympathetic stimula tion which in turn is likely to continue after cessation of CO_2 causing an over shooting The same phenomenon may also affect myocardial contractile force and cardiac output so that changes in cardiac output and the adjustment of the blood pressure are supposed to be contingent on each other Finally sinus arrhythmia which in this study markedly intensified as pCO_2 increased clearly caused a corresponding fluctuation both in cardiac output and in arterial blood pressure

When arterial pCO_2 was increased from hypo to normocapnia most of the changes in cardiovascular factors were parallel to those accompanying an increase from normo- to hypercapnia In most cases an increase was seen in arterial pressure stroke volume and right atrial pressure while heart rate slowed and sinus arrhythmia became more prominent The inference is that changes in carbon dioxide tension exert stimuli which cause similar changes in the hypocapnic normal and hypercapnic states

A release of catecholamines from the adrenal medulla has been suggested to be responsible for the rise in cardiac output during CO_2 breathing (Nahas *et al* 1960 Ligou and Nahas 1960) It has been reported on the other hand that low CO_2 concentrations up to 8 per cent in the inspired air have no catecholamine releasing effect (Tenney 1956 Honig and Tenney 1957 Manley *et al* 1964) In the present study cardiac output increased markedly in some cases while in other cases a clear decrease was observed This was also seen in the hypocapnic state where the adrenals are not stimulated Hence it cannot have been the catecholamines that were responsible for the cardiac output changes observed in this study

In decreasing pCO_2 most of the changes were the reverse of those seen during increasing pCO_2 However increased pCO_2 caused a rise in TPR in 73 per cent of experiments while a lowering of pCO_2 decreased TPR in only 53 per cent In the study taken all in all increasing pCO_2 caused a significant rise in TPR while lowering pCO_2 led to no significant change in TPR The sympathetic stimulus seemed to last longer than the local vasodilating effect of CO_2 so that the change caused by an increase of pCO_2 continued after pCO_2 had been reduced to the control level

The fluctuations in arterial pressure and cardiac output which were observed at higher pCO_2 values diminished on lowering pCO_2 and were completely abolished in hypocapnia The situation was almost the same with sinus arrhythmia In hypercapnia it was markedly stronger than in normocapnia and mostly disappeared when pCO_2 was reduced below normal

In contrast to the disturbances in cardiovascular homeostasis observed during rising pCO_2 and hypercapnia no marked disturbances could be seen during reduction of CO_2 tension or in hypocapnic states but on the whole circulatory homeostasis seemed to be stabel and easily maintained

It can be stated that there are several constant phenomena in the cardiovascular response to changes in arterial carbon dioxide tension during thoracotomy The cardiovascular reactions are influenced by other partly unknown factors however so that the final answer especially regarding changes in cardiac output has not yet been found In various reports concerning changes in cardiac output due to variations in arterial pCO_2 great differences can be seen in the consistency and direction of the changes The same was observed in this study Many different factors which adjust cardiac output are affected by changes in pCO_2 so that under the experimental conditions prevailing in this study changes in cardiac output are inconsistent and unpredictable

In clinical evaluation of the effects of changing pCO the slowing of heart rate and severity of sinus arrhythmia observed with higher pCO_2 values has to be taken into consideration Although no severe cardiac irregularities were seen in this study surgical manipulation especially during cardiac surgery may lead to more severe conditions Sudden change in arterial pCO_2 and especially sudden rise in carbon dioxide tension disturbes often cardiovascular homeostasis Hypercapnia during surgery is often accompanied by hypoxia which augments the risk of cardiac dysfunction

Hypocapnia seemed not to lead to any clinically harmful changes in the cardiovascular functions examined in this study However the blood flow to certain organs especially the brain decreases markedly when arterial carbon dioxide tension is lowered to subnormal values Anaesthesia and surgery may potentiate some unwanted

effects. Blocking of the regulating cardiovascular reflexes may lead to dramatic decreases in cardiac output.

In view of previous findings it is preferable to avoid both hypercapnia and hypocapnia. The best conditions for tolerating anaesthesia and surgery will be afforded to the cardiovascular system by keeping arterial carbon dioxide tension within normal limits and by avoiding sudden changes in CO_2 tension.

VII SUMMARY AND CONCLUSIONS

The aim of the present investigation was to study the cardiovascular response to changes in arterial carbon dioxide tension at different levels of increasing and decreasing pCO_2 during thoracotomy

The study was performed on dogs under very light thiopental anaesthesia and controlled ventilation Experiments were made on altogether 35 dogs and several experiments were performed on most of the dogs. Thoracotomy was done through the fourth right intercostal space Ventilation was kept constant with a respirator throughout the study 100 per cent oxygen was used as respiratory gas and the effects of CO_2 were studied by using 5 per cent CO_2 in oxygen

Cardiovascular parameters were first recorded under 100 per cent oxygen and again under ventilation with 5 per cent CO_2 when a stable state was reached Finally the inhaled gas mixture was restored to 100 per cent oxygen and the findings recorded

During the study cardiac output was determined by means of an electromagnetic flowmeter placed around the ascending aorta Heart rate was calculated from recorded ECG which was also used to observe changes in cardiac rhythm Arterial and right atrial pressure were measured electronically stroke volume and total peripheral resistance were calculated from the readings obtained

The following conclusions can be made

Heart rate

Changes in heart rate definetely and significantly followed changes in arterial carbon dioxide tension This was clearly observed between all three different groups under investigation hypercapnia normocapnia and hypocapnia The correlation between changes in pCO_2 and heart rate were highly significant during both increasing and decreasing pCO_2 Only in very few cases was there no change in heart rate or a change in the opposite direction to the pCO_2 change The fact that vagotomy does not abolish the regulating effect of CO indicates that in addition to the vagal stimulus there are other mechanisms controlling heart rate Carbon dioxide affects these either directly or by changing the hydrogen ion concentration

Mean arterial pressure

As a whole changes in mean arterial pressure are correlated with corresponding changes in pCO_2 A rise in carbon dioxide tension is followed by an elevation in arterial pressure and vice versa In smaller more detailed groups the results were not so uniform and variations could be seen in most smaller groups Fluctuations in arterial pressure were observed after the inspired gas was changed

from oxygen to 5 per cent CO_2 or back to oxygen These seem to be due to sudden imbalance between local vasodilatation and central sympathetic stimulation caused by CO_2 Changes in cardiac output also produced changes in arterial blood pressure

Total peripheral resistance

Total peripheral resistance was calculated as a ratio of mean arterial pressure to cardiac output Since both factors change during the experiment there is a risk of inaccuracies in quantitative measurement but under controlled conditions it is possible to discern the direction of changes in TPR

In the combined results the correlation between total peripheral resistance and arterial pCO_2 was significant When changes in TPR were studied separately in increasing and decreasing arterial pCO_2 the differences were greater Increasing pCO_2 significantly raised the total peripheral resistance but reduction of arterial carbon dioxide decreased TPR only in 53 per cent There appeared to be variations in the local vasodilatative action of CO_2 and in the occurrence and persistence of central stimulation caused by this agent with resultant variations in TPR during the reduction of pCO_2

Cardiac output

Changes in cardiac output are not correlated with alterations in arterial pCO_2 A change in the inhaled gas mixture caused unpredictable changes in cardiac output both increase and reduction being observed In the initial stage after changing the gas mixture a fluctuation in the cardiac output lasting several minutes could bee seen before the more stable phase was reached This is apparently due to an imbalance between different components in the regulation of cardiac function The adjustment of cardiac output depends on so many different factors that changes in cardiac output cannot be estimated on the basis of the findings made in the present study

Stroke volume

The stroke volume in the present study was calculated by dividing cardiac output by heart rate

In contrast to the cardiac output changes in stroke volume were significantly correlated with arterial pCO_2 increasing pCO_2 mostly elevated stroke volume and vice versa This correlation could be seen during both increasing and decreasing CO_2 tension It was seen at all stages of arterial pCO_2 in hypercapnia as well as in normo and hypocapnia

Stroke volume was also correlated with heart rate When the heart rate was slow the stroke volume was greater and with accelerating heart rate it diminished

Right atrial pressure

Thoracotomy raises the extracardial pressure which also elevates the pressure in the right atrium A rise in arterial CO_2 tension concomitantly elevated right atrial pressure In overall study the correlation between CO_2 and RAP was highly significant The same was true in most sub groups Increasing pCO_2 elevated right atrial pressure while decreasing pCO_2 lowered it The situation was similar in hyper normo and hypocapnia

Cardiac rythm

The dog's heart seems to tolerate hypercapnia and changes in carbon dioxide tension extremely well. Severe arrhythmias were not observed during the experiments performed, either during hypercapnia or during the posthypercapnic period. A frequently observed finding was sinus arrhythmia, which was accentuated by hypercapnia. It became less marked during falling pCO_2 tension and often disappeared completely in the hypocapnic state. Other irregularities in heart rhythm were sinus arrest, observed in six experiments, and nodal rhythm, seen in one. It is believed that vagal stimulation during hypercapnia causes a greater response in the rhythm controlling system than in lower pCO_2 tension. During hypocapnia the phasic vagal effect seemed to be negligible.

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APPENDIX

Table 13 Heart rate standard deviation of R R time and standard deviation of R R time as percentages of the mean $\left(100\ \frac{SD_{R\ R}}{\text{mean R R}}\right)$

Dog no	pCO_2	HR	$SD_{R\ R}$	$SD_{R\ R}\%$
1	26.8	210	0.00	0
	48.6	180	0.23	2
	48.7	180	0.20	2
2	21.2	218	0.00	0
	22.2	206	0.00	0
	25.7	210	0.25	3
	32.5	206	0.00	0
	39.4	164	0.[illegible]	7
	41.3	160	0.57	5
	42.3	156	0.93	9
	49.1	130	1.73	14
4	36.2	185	0.00	0
	39.0	210	0.23	3
	41.1	195	0.00	0
	52.5	132	0.96	7
	60.0	120	0.63	5
5	59.4		1.91	18
[illegible]	28.8	194	0.20	2
	40.5	196	0.24	3
	54.0	152	0.70	6
7	30.6	224	0.00	0
	39.3	210	0.21	2
	40.0	210	0.00	0
	61.4	110	1.58	11
8	26.3	150	0.48	5
	53.0	138	0.86	7
9	34.5	146	0.17	2
	37.0	145	0.00	0
	44.5	105	0.00	0
	45.7	112	0.36	2
	55.5	98	1.05	6
10	29.8	170	0.45	5
	36.5	140	1.30	11

(CONT.)

(CONT)

Dog no	pCO_2	HR	$SD_{R\,R}$	$SD_{R\,R}$°
11	31 5	210	0 00	0
	39 0	214	0 25	3
	39 3	210	0 20	3
	5[illegible] 7	200	0 12	[illegible]
	60 8	174	0 45	5
	65 0	168	1 [illegible]8	14
12	[illegible]8 4	156	0 37	3
	32 8	155	0 47	5
	41 3	138	0 72	6
13	36 0	154	1 18	10
	38 0	176	0 65	[illegible]
	55 7	135	1 93	15
14	45 0	220	0 00	0
	66 1	190	0 25	3
19	29 3	170	0 00	0
	30 1	170	0 00	0
	34 4	170	0 35	4
	55 0	1[illegible]0	2 01	19
22	40 0	124	2 05	17
	45 7	118	2 86	23
	61 0	100	3 66	25
24	42 5	184	0 00	0
	45 0	194	0 00	0
	45 2	180	0 29	3
	71 4	130	[illegible] 33	18
	77 3	100	3 29	22
25	40 4	1[illegible]0	1 90	15
	60 5	110	3 14	25
28	43 4	14[illegible]	0 18	2
	53 0	144	0 68	[illegible]
[illegible]9	36 1	116	1 04	8
	53 0	124	1 48	13
30	3[illegible] 3	174	0 00	0
	36 8	180	0 25	3
	55 2	132	0 7[illegible]	7

(CONT)

(CONT)

Dog no	pCO_2	HR	SD_{R-R}	SD_{R-R} %
31	28 4	176	0 00	[illegible]
	36 1	155	0 25	2
	44 8	154	0 25	3
	67 2	120	0 96	7
32	46 1	180	1 73	19
	56 0	182	0 67	8
33	35 4	180	0 22	3
	70 1	120	2 20	18
34	41 9	120	1 89	14
35	38 5	154	0 63	[illegible]
	70 0	150	0 50	5

Table 14 Detailed results of the experiments

Exp	pCO_2	Heart rate	R R interval	Cardiac output	Stroke volume	Mean arterial press	Periph resist	Right atrial press	pH	pO_2
I	62 5			8 3		106	12 9		7 09	
	48 7	180	0 34–0 36	8 0	445	104	13 0		7 18	
II	_6 8	_10	0 30–0 30	6 6	314	100	15 2		7 12	
	48 6	180	0 34–0 36	7 8	433	110	14 1		7 18	
I	32 5	206	0 30–0 30						7 38	198
	21 2	218	0 28–0 28	6 2	284	112	18 1		7 40	200
	39 4	164	0 36–0 44	7 8	476	134	17 2		7 18	191
	49 1	130	0 40–0 64	7 0	537	136	19 4		7 16	214
II	45 0	126		6 7	532	124	18 5		7 18	
	25 7	210	0 28–0 30	6 2	295	126	20 3		7 33	242
III	23 2	206		5 8	281	110	19 0		7 36	232
	41 3	160	0 38–0 44	6 2	388	130	21 0		7 18	_09
	22 2	206	0 30–0 30	6 0	291	120	20 0		7 37	223
	42 3	156	0 36–0 48	6 4	410	1_5	19 5		7 18	190
	50 1	125							7 17	
	16 8	145						0	7 45	330
	66 4	120						1	6 84	
I	39 0	210	0 28–0 30	6 2	295	132	21 3	7	7 28	>200
	33 6	210		5 8	276	136	23 4	6	7 30	185
	52 5	132	0 48–0 56	5 2	394	130	25 0	7	7 _0	>_00
	36 2	185	0 34–0 34	6 0	374	136	2_ 6	6	7 27	
	60 0	120	0 46–0 5_	6 5	541	145	22 3	7	7 13	
	41 1	195	0 32–0 32	5 8	297	136	23 5			
	52 8	120		3 5	291	98	28 0		7 22	
	42 7	120		4 1	341	95	23 2		7 31	
	38 9	142		4 3	310	100	23 3		7 34	
	59 4		0 36–0 56						7 14	
	28 8	194	0 32–0 34	6 6	340	112	17 0		7 22	253
	54 0	152	0 44–0 52	6 8	448	126	18 6		6 99	291
	40 5	196	0 32–0 34	6 4	326	118	18 4		7 20	210
I	39 3	210	0 28–0 30	4 3	204	155	36 0	0	7 27	245
	30 6	224	0 28–0 28	4 0	178	150	37 6	−1	7 29	216
	57 0	120		4 3	368	140	32 6	−1	7 13	210
II	61 4	110	0 48–0 64	4 0	364	136	34 0	−1	7 15	194
	40 0	210	0 28–0 _8	4 _	200	152	36 2	−1	7 21	169

(CONT)

(CONT)

Dog no	Exp	pCO_2	Heart rate	R R interval	Cardiac output	Stroke volume	Mean arterial press	Periph resist	Right atrial press	pH	pO_2
8	I	26 3	150	0 40–0 46	5 8	387	118	20 4	0	7 35	>400
		56 0	140		5 4	386	160	29 6	3	7 22	400
	II	53 0	138	0 42–0 56	5 9	427	140	24 7	2	7 18	384
		38 2	155		5 4	360	132	24 4	0	7 26	400
9	I	34 5	146	0 42–0 44	4 9	345	96	19 6	3	7 34	235
		37 0	145	0 42–0 42							
		55 5	98	0 60–0 72	4 5	474	110	24 4	4	7 13	
		45 7	112	0 56–0 60	3 8	339	100	26 3	2	7 20	
	II	42 8	104		3 5	336	100	28 6	2	7 25	
		59 4	96		3 5	365	100	28 6		7 15	
		44 5	105	0 60–0 60	3 4	324	102	30 0	2	7 23	
10		36 5	140	0 40–0 56	4 2	300	136	32 4	−2	7 27	
		29 8	170	0 36–0 40	4 7	277	136	29 6	−4	7 28	
11	I	39 3	210	0 28–0 30						7 19	
		31 5	210	0 30–0 30						7 24	
	II	39 0	214	0 28–0 30						7 12	
		60 8	174	0 34–0 40						7 07	
		65 0	168	0 32–0 48						7 05	
		52 7	200	0 30–0 32						7 11	
12	I	28 4	156	0 40–0 44						7 15	
		32 8	155	0 40–0 44	4 5	300	130	28 8	−1		
		45 0	100		4 3	430	160	37 2	1		
	II	41 3	138	0 40–0 52	3 7	268	136	36 8	0	7 09	
		50 3	110		3 8	346	145	38 2	2	7 02	
		47 0	126		3 6	286	116	32 2	1	7 05	
13		36 [illegible]	154	0 36–0 56			120		4	7 33	
		55 7	135	0 42–0 68					4	7 15	
		55 0	140							7 18	
		38 0	176	0 34–0 44			115		[illegible]	7 32	
14		66 1	190	0 32–0 34	5 6	295	118	21 1		7 11	
		45 0	220	0 28–0 [illegible]	5 0	228	104	20 8		7 19	
15		33 4	130		6 0	461	100	16 7	3	7 27	
		59 2	115		5 8	504	110	19 0	5	7 04	
		44 7			5 5		104	18 9	4	7 18	

(CONT)

og o	Exp	pCO_2	Heart rate	R R interval	Cardiac output	Stroke volume	Mean arterial press	Periph resist	Right atrial press	pH	pO_2
6		36 1	132		4 3	326	144	33 5	3		
		63 5	1_0		3 2	266	134	41 9	7	7 15	
		39 1	1_0		4 0	334	140	35 0	4	7 26	
7		20 9			2 2		125	56 9	7	7 40	
		47 4			2 0		125	62 5	10	7 19	
		_9 8			2 4		125	52 1	7	7 27	
8		58 9	126				126			6 98	117
		47 9	124				1_6			7 09	121
9		79 3	170	0 34—0 34							
		30 1	170	0 34—0 34	4 3	253	150	34 9	6		
		55 0	120	0 36—0 60	3 5	292	170	48 5	9		
		34 4	170	0 32—0 38	3 8	223	145	38 2	8	7 34	
0	I	48 0			3 4		132	38 8	0	7 23	
		63 4			3 0		134	44 4	0	7 08	
	II	50 3			5 5		144	26 2	2	7 16	
		44 5			5 7		140	24 6	2	7 29	
21		57 6	120		4 5	376	130	28 9	4	7 08	280
		31 7	140		4 8	342	130	27 1	2	7 26	
22		40 0	124	0 40—0 72	6 0	485	130	21 7	7		
		61 0	100	0 44—0 96	4 0	400	100	25 0	8	7 08	
		45 7	118	0 40—0 80	6 0	510	110	18 4	6	7 20	
23	I	64 0	112		4 5	402	124	28 0	2	7 14	336
		46 4	150		4 2	280	120	28 6	1	7 16	
	II	45 5	150		4 5	300	125	27 8	1	7 25	
		75 9	146		5 0	343	140	28 0	3	7 07	
		44 9	158		4 6	291	125	27 2	0	7 22	
24	I	77 3	100	0 44—0 84	3 8	380	120	31 6	10	7 09	
		45 2	180	0 34—0 38	4 1	228	120	29 3	6	7 20	
	II	45 0	194	0 34—0 34	4 0	206	120	30 0	4	7 20	
		71 4	130	0 40—0 80	3 8	292	130	34 2	9	7 11	
		42 5	184	0 34—0 34	3 7	201	120	32 5	6	7 28	
25		40 4	120	0 44—0 64			150		8	7 34	
		60 5	110	0 40—0 76			150		11	7 24	
		45 0					160		9		

(CONT)

Dog no	Exp	pCO_2	Heart rate	R R interval	Cardiac output	Stroke volume	Mean arterial press	Perich resist	Right atrial press	pH	p
26		54 5	126				160		6	7 10	
		37 5	126				150		4		
27		34 8	90				145		6	7 37	
		37 8					160			7 33	
28		53 0	144	0 40—0 48			130		[illegible]	7 33	
		43 4	142	0 44—0 46			128		6	7 35	
29		53 0	124	0 40—0 60					6	7 22	
		36 1	116	0 44 0 60					5	7 35	
30		32 3	174	0 34—0 34	3 7	212	152	41 5	7	7 35	
		55 2	132	0 38 0 46	3 9	295	180	46 2	12	7 18	
		36 8	180	0 34—0 36	3 4	189	150	44 1	6	7 33	
31		44 8	154	0 38—0 40							
		28 4	176	0 36 0 36	5 0	285	150	30 0	4	7 33	
		38 9	150		4 9	327	155	31 6	4	7 22	
		67 2	120	0 44 —0 64	4 0	333	155	38 8	12	7 13	
		36 1	155	0 38—0 40	5 0	322	150	30 0	4	7 27	2
32		56 0	182	0 30 0 40	5 5	303	165	30 0	3	7 14	
		46 1	180	0 30—0 52	4 8	267	150	31 2	0	7 35	
33		35 4	180	0 32—0 34			180		0	7 40	
		70 1	120	0 38 0 72			170		7	7 17	
		35 7	118				170		3		
34		41 9	120	0 46 0 68							
		39 1	120				110		4	7 33	
		73 8	100				135		8	7 09	
		45 0	120				120		5		
35		38 5	154	0 36—0 44			150		1	7 38	
		70 0	150	0 36—0 44			164		3	7 10	17
		40 [illegible]					150		1		

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Microfluorometric Characterization of Intracellular Nucleic Acids and Nucleoproteins by Acridine Orange

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Symbols and Abbreviations used in the text

Symbols

α = ratio of the uncorrected fluorescence intensities at 90 nm which is a function of the acridine orange nucleic acid p and thus a measure for the degree of dye aggregation on

B = radiant density or brightness of a light source [candela/cm

C = ratio of the fluorescence coefficients of high order (double (single stranded) nucleic acid AO complexes at 530 nm (f

E = extinction or optical density

E_{tot} = total extinction i.e. the surface integral of the extinction ($\int_{\lambda} E d\lambda [\mu]$)

F = fluorescence intensity (given in mV amplified photomultipl

f = fluorescence coefficient i.e. fluorescence intensity per 10 phorus [mV/10 mole P]

I = radiant energy per unit time (intensity) of a luminescent ob

J = intensity of irradiation or illumination [lumen/cm]

k = absorption coefficient

λ = wavelength in nanometer [nm = 10 m]

μ = ionic strength of a buffer solution

μm = μ [10 m]

n = refraction index

ν = wavenumber [cm]

p = degree of polarization

Q = quantum yield of fluorescence

Φ = optical path difference [cm]

Ψ = specific optical path difference [cm /g]

W = radiant energy per unit time of a light source equivalent

Abbreviations

AO = Acridine orange

DNA = Deoxyribonucleic acid

DNP = Deoxyribonucleoprotein

PHA = Phytohemagglutinin

Poly A = Polyadenylic acid

Poly U = Polyuridylic acid

RNA = Ribonucleic acid

RNP = Ribonucleoprotein

Introduction

Microspectrophotometry, which has been pioneered by Caspersson (1936, 1950) has become an invaluable tool for the analysis of cell components and their functions. It differs from other quantitative methods in permitting the study of the life processes of individual cells and of their distribution within the varying spectrum of a cell population. It can also provide information about the chemical and physicochemical properties of cell substances in their original condition and localization, information which may prove to be different from data obtained in the isolated state.

In spite of these advantages and the possibilities which microspectrophotometry offers, only a limited number of quantitative methods have been developed, apart from the almost classical techniques for the determination of nucleic acids and proteins from the UV absorption and of DNA from the Feulgen reaction. This is mainly because the quantification of a chemical reaction at cell level likewise has to be done at the microlevel if accurate results are to be obtained: quantitative *in vitro* relationships between biological substances and their reaction products do not imply that such results are directly applicable for the microdetermination of intracellular substances occurring in high concentrations with heterogeneous distribution. Thus the investigation of staining reactions at cell level calls for a variety of complex instruments such as rapid scanning microspectrophotometers and microinterferometers (Caspersson and Lomakka, 1962) and these were not developed until recent years.

One of the most interesting of the wide range of staining reactions seemed to be that obtained with acridine orange (AO). Leaving aside the beauty of a number of biological structures when stained with acridine orange there is the rapid application of this technique in medical and other biological fields for the detection and identification of intracellular nucleic acids. The volume of information concealed in the metachromatic phenomena exhibited by acridine orange binding to nucleic acids served as a direct challenge to put this staining reaction on a quantitative basis.

Acridine orange was first introduced as a stain or fluorochrome for plant structures by Bukatsch and Haitinger (1940) and was adopted later for microorganisms by Strugger (1949). Its value for the staining of animal cells was

demonstrated by Schummelfeder (1950, 1957) Armstrong (1956) and Bertalanffy and Bickis (1956) The work of these authors indicated that the orthochromatic green fluorescence was due to DNA and the metachromatic red fluorescence to RNA The ease and elegance of this method has made it the dominant staining reaction for the detection and identification of intracellular nucleic acids

The first explanation of the metachromatic behaviour of acridine orange and with it the basis for further investigation of the stoichiometry of the staining reaction was provided by Zanker (1952) He demonstrated that the metachromatic shift exhibited by the dye was due to an association of the dye molecules at increasing dye concentrations As a consequence of this work and the highly interesting results obtained from staining biological structures with acridine orange a number of basic investigations were made into the interaction between acridine orange and nucleic acids (Morthland *et al* 1954 Steiner and Beers 1957 Bradley and Wolf 1959) Surprisingly enough DNA AO and RNA AO complexes which exhibit such striking differences in AO stained biological structures were hardly distinguishable by their spectral behaviour in solution Until Bradley and coworkers reported a difference in the binding affinity of AO to DNA and RNA respectively it was generally believed that DNA and RNA are virtually indistinguishable in this respect

These partly conflicting results are explainable, however, by the fact that DNA AO and RNA AO complexes exhibit their largest spectral differences when the binding sites of the nucleic acids are saturated according to their affinity for AO Differences in the dye affinity are best revealed by staining the nucleic acids with an excess of AO followed by a dialysis of the unbound or incompletely bound dye If on the other hand the nucleic acids are added to AO solutions as in the case of nucleic acid-AO complexes in solution the AO binding is primarily a function of the ratio of dye to nucleic acid binding sites small differences in the affinity of AO to DNA and RNA binding sites would then be concealed

In the present study therefore the dye binding properties of nucleic acids and nucleoproteins were investigated by spraying these onto glass slides and staining the resultant microdroplets of cell size in an excess of AO followed by dialyzation This approch was made possible by the development of a highly sensitive microspectrofluorometer (Caspersson *et al* 1963) described and discussed in Chapter 2 The results demonstrate that the binding of AO is dependent on the nucleic acid structure as well as on the protein nucleic acid interaction Chapters 3 and 4) An investigation of the kinetics of the AO binding to intracellular nucleic acids is reported in Chapter 5 The practical application of the AO technique for quantitative determination of intracel

lular nucleic acids and nucleoproteins which is distinguished by its high sensitivity is explained in Chapter 6 In the last section of the same Chapter the various possibilities of this technique are demonstrated in a study of the molecular changes of the deoxyribonucleoprotein complex in different stages of cell function

Materials and Methods

A. Materials

1. Nucleic acids

The nucleic acid fractions used in the investigation of the acridine orange binding were a high polymer DNA isolated from calf thymus after Zamenhof 1957 (Sigma Chem. Co., Type I), RNA isolated from yeast ribosomes after Crestfield *et al.* 1955 (Sigma Chem. Co., Type IX), Poly U (S_{20} = 7.15) and Poly A (S_{20} = 8.74) (Miles Chem. Co.). The N/P ratio (nitrogen determined after Kjeldahl, phosphorus after Teorell 1931) was 1.7 for DNA, 1.8 for RNA, 1.2 for Poly U and 2.5 for Poly A; the extinction coefficients per mole of phosphorus at 260 nm (ε^P_{260}) were 7200, 9750, 10000 and 9300 respectively.

Each fraction was dissolved with equal parts of gelatine (DIFCO Lab.) in bidistilled H_2O and sprayed with a glass spray flask onto optically parallel glass slides (20 × 26 × 0.3 mm coverslips for hemocytometer chambers "Bürker" slides) from a distance of about 30 cm using a pressure of ca. 2.5 kg/cm². Pure nitrogen was employed as the spray gas to avoid changes in the nucleic acid resulting from exposure to oxygen at high pressure. All nucleic acids were sprayed at a concentration of 25 mg/ml which gave microdroplets 2–100 μm in diameter. Gelatine was used as a relatively inert carrier in order to avoid elution of the nucleic acids from the droplets during the staining procedures.

2. Nucleoproteins

The deoxyribonucleoprotein fractions were isolated according to Zubay and Doty 1959 from calf thymus and the ribonucleoprotein fractions according to Kirsch, Siekevitz and Palade 1960, 1962 from rat liver employing 0.3 per cent deoxycholate. They were sprayed in a concentration of 10—30 mg/ml without gelatine in the same manner as the nucleic acid fractions. The N/P ratio averaged 3.9 for DNP and 4.1 for RNP. The purity of the ribosome fractions was also controlled by electron microscopic examination of the isolated material sprayed on copper grids and fixed in glutaraldehyde followed by 2 per cent phosphotungstic acid.

3 *Mouse fibroblast cells*

The mouse fibroblast cultures used for investigating the staining kinetics were a constantly growing tetraploid cell line of the type L-929 The cells were cultivated in Eagle s medium with 10 per cent calf serum at 37 C for 48 and 72 hours as monolayers on Burker slides For the biochemical determination the nucleic acids were extracted with 5 per cent trichloro-acetic acid for 15 minutes at 90 C (Schneider 1945) DNA was determined with diphenylamine according to Dische as modified by Burton (1956) RNA with orcinol according to Mejbaum (1939)

4 *Human leucocytes*

The human leucocytes were obtained from the peripheral blood of healthy donors Several aliquots of blood (10 ml) were each mixed with 0 1 ml of heparin (1,000 IE/ml) and 0 5 ml of III per cent dextran (M_w = 153 000) dissolved in 0 9 per cent NaCl This mixture was allowed to stand for 30 minutes at + 4 C Thereafter the plasma was centrifuged at 1 800 rpm (350 × g) for 7 minutes The supernatant was discarded and the cells thus obtained were resuspended in autologous dextran free plasma This cell suspension was then diluted 1 4 with Eagle s medium and the heparin concentration was adjusted to a final concentration of 5 IU/ml The cell suspension was prewarmed to 37 C and inoculated into plastic Petri dishes containing Burker or quartz slides Bacto PHA P (DIFCO) rehydrated with 5 ml of sterile distilled water diluted 1 9 with 0 9 per cent NaCl was used in the proportion of 0 1 ml per 5 ml of the cell suspension After various incubation times at 37 C the slide cultures were washed in 0 9 per cent NaCl as were the mouse fibroblast cultures and fixed as stated below

5 *Spermatozoa*

The human and bull spermatozoa were obtained from the ejaculate and from testicle scrapes and washed three times in a buffered balanced salt solution (Mann 1964) The sediment was smeared on Bürker and quartz slides and subsequently fixed

6 *Bacteriophages*

Sample T_2 sprayed in a concentration of 10^{14} particles/ml physiological saline onto Burker slides was kindly provided by Professor G Bertani Karolinska Institutet

7 *Human chromosomes*

Human chromosomes were obtained from PHA stimulated human lymphocyte cultures after incubating the cells for 3 hours with Colcemide. After hypotonic shock in 0.8 % Na citrate for 15 minutes and fixation in ethanol : acetic acid (3 : 1) for 30 minutes the cells were squashed in 10—50 per cent acetic acid with a "Vipolon" covership which was dissolved by acetone (Östergren and Heenen 1962).

B Methods

1 *Fixation*

The nucleoprotein fractions and all cells, except the chromosome preparations, were fixed for 30 minutes at room temperature in a mixture of ethanol : acetone in a ratio of 1 : 1 (v/v) and kept up to 24 hours at + 4° C in the same mixture before staining. The nucleic acid gelatine specimens were fixed in formaldehyde gas for 10 minutes (to prevent a loss of nucleic acids due to swelling of the gelatine in water) and stained immediately. Whereas nucleoprotein fractions and cell specimens could be kept for no more than 72 hours and only in the fixation mixture at + 4° C without any noticeable change to the dye binding, no alteration of the dye affinity to nucleic acid fractions was observed when these were stored over a period of several months in vacuo at + 4° C. For freeze substitution, which was used in some experiments, the specimens were immersed in liquid propane at — 196° C and subsequently dehydrated with absolute ethanol at — 70° C. The temperature rise from — 70° C to room temperature was extended over a period of at least 72 hours.

2 *Staining with acridine orange*

Staining was performed in a thermostatic waterbath (Fig. 1) with a tolerance of 0.1° C for the temperature of the staining solution. This condition is important for a quantitative evaluation of an ion binding reaction (see Chapter 5). The glass slides with the specimens were placed vertically and 2 mm apart in holders made from polyethylene or porcelain (Thomas Co., USA) and immersed in the staining solutions as follows:

1)	Pyridine water free	5 minutes
2)	Pyridine : acetic acid anhydride (3 : 2, v/v)	15 minutes
3)	Ethanol water free	5 minutes
4)	Ethanol 96 per cent	5 minutes
5)	Ethanol 60 per cent	5 minutes

6)	Ethanol 30 per cent	5 minutes
7)	H_2O redistilled	3 minutes
8)	Citric acid Na_2HPO_4 buffer	5 minutes
9)	Citric acid Na_2HPO_4 buffer + acridine orange	15 minutes
10)	Citric acid Na_2HPO_4 buffer	5 minutes
11)	Citric acid Na_2HPO_4 buffer	5 minutes
12)	Citric acid Na_2HPO_4 buffer	5 minutes

Steps 1 and 2 were omitted in the experiments without acetylation and step 8 in the staining of nucleoproteins The staining of the nucleic acid fractions after fixation with formaldehyde gas started with step 9 the staining time being shortened to 10 minutes Within the total time of 25 minutes the elution of nucleic acids from the droplets detected by dry mass determination of the microdroplets before and after incubation in pure buffer solution was less than 5 per cent The same was true for the nucleoprotein fractions when kept no longer than 30 minutes in citric acid Na_2HPO_4 buffer

All reaction vessels (made from pyrex glass with a capacity of 200 ml) were filled with 100 ml of each reaction solution and placed in a vibration free thermostat These volumes are important especially for steps 10 11 and 12 since the diffusion speed of the dye ions varies with the concentration gradient The entire staining procedure was performed at 20 C to diminish the activity of nucleic acid splitting enzymes Furthermore the use of citric acid in the buffer mixture inactivates DNases by precipitating the necessary bivalent ions After the final step, the preparations were kept in a vertical position to drain off the excess buffer solution before covering with citric acid phosphate buffer of the same pH and a coverslip (18 × 18 mm) sealed with paraffin of a low melting point (mp 52 C)

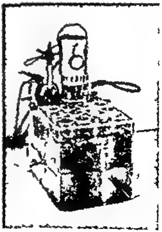
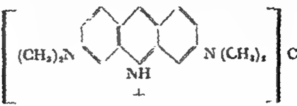


Fig 1 The thermostatic apparatus for AO staining

3. *Acridine orange*

Acridine orange (3,6-bis-dimethyl-amino-acridinium chloride, AO)



(Merck C. I. 46005, S. No. 902) was separated from associated impurities using an Al_2O_3 column (Zanker, 1952a) and recrystallized three times from chloroform and ether. Its purity was checked by determining the melting point (mp 180—181° C) and the molar extinction coefficient at 490 nm (55000, dye concentration 10^{-5} M). It was kept in a stock solution in a concentration of 1 mg/ml redistilled H_2O for a maximum of 4 weeks at + 4° C and added to the buffer solution to give a final dye concentration of 10^{-4} M.

4. *Measuring procedures*

All measurements were made immediately after staining and were finished within 24 hours at the latest. No variations in the measured values could be observed during this time when the specimens were kept at + 4° C.

a) *Absorption spectra*

The absorption spectra were registered with an UMSP I microspectrophotometer (Caspersson *et al.* 1953, 1954, 1966) (objective: Zeiss Achromat 100 ×/N. A. 1.25, condenser: Zeiss UV Achromat 30 ×/N. A. 0.4, measuring point 1.0 µm in diameter, speed of the automatic wavelength scan ≈ 46 cm^{-1}/sec.)

b) *Emission spectra*

The emission spectra were registered with a microspectrofluorometer constructed at this institute (Caspersson, Lomakka and Rigler Jr., 1965) (objective: Reichert 100 ×/N. A. 1.25 -UV- with a built in Schott GG 9 filter before the front lens to cut off exciting radiation; condenser: Zeiss Ultrafluar 100 ×/N. A. 0.85) using a constant slit width of 0.2 mm. The resulting band width at 530 nm was 8.6 nm (quartz prism monochromator Zeiss PMQ II). The diameter of the measuring point was varied between 1 and 10 µm in order to register with a constant slit width. The registration speed of the automatic wavelength scan was ≈ 50 cm^{-1}/sec. The receiver used was a RCA 1P 28 photomultiplier. A calibrated tungsten band lamp of 2700° color temperature was used as standard energy source (de Vos, 1953) to correct the emission spectra

for wave length dependent changes of the photomultiplier sensitivity and of the transmission of the optical system All emission spectra in the text have been corrected if not stated otherwise The objects were excited to luminescence at 365 nm using a slit width of 1 8 mm (band width 25 nm) Since the absolute height of the absorption as well as of the emission spectra varies with the thickness of the microdroplets and the nucleic acid concentration of the cells, only the relative spectral distribution is given, taking the maximal value recorded as unity The measuring points of the emission spectra however were correlated to the dry mass or the phosphorus content of the nucleic acid droplets at the two wave lengths 530 nm and 590 nm

c) *Emission measurements*

Two types of optical arrangements were used

In order to get a reliable mean for large cell populations in a short time—as in the investigation of the kinetics of the acridine orange staining of biological material—several cells were measured together, using a low magnification (objective Reichert 63 ×/N A 0 75-UV-) and a large measuring field (110 μm diameter, condenser Zeiss UV Achromat 30 ×/N A 0 3) Since measurements were made of 3 specimens per step, with 10 areas having about 4 to 5 cells each per specimen the mean fluorescence intensity of about 150 cells could be obtained very quickly Single cells or microdroplets were measured with a high magnification (objective Reichert 100 ×/N A 1 25-UV-) and a small measuring field (60 μm diameter for microdroplets and mouse fibroblast cells 20 μm and less for lymphocytes and single chromosomes condenser Zeiss Ultrafluar 100 ×/N A 0 85)

The same slit width was always used for quantitative measurements i e 0 2 mm of the second monochromator analyzing the emittent radiation, with a standard system for calibration of the exciting radiation (see Chapter 2) The sensitivity of the detecting system was extended by raising the multiplier voltage in the case of very weak light intensities and by placing calibrated neutral glass filters before the second monochromator in the case of light intensities in excess of the amplifier s normal sensitivity range All the emitted light intensities reported here in mV amplified photomultiplier signal are thus related to a constant excitation energy and a constant slit width of 0 2 mm of the second monochromator To correct for incompletely filtered off exciting radiation and fluorescence contributed by the optical system the background fluorescence intensity of every specimen has been subtracted from its own fluorescence intensity While all intensities of the emission spectra have been corrected for wave length dependent changes of the photomultiplier sensitivity and of the transmission of the optical system in order to permit a

were computed with an IBM 1401 electronic computer. Each calculated final value is the mean of at least two successive experiments.

The DNA phosphorus contents of spermatozoa and lymphocytes (see Chapter 6) were determined from the UV absorption at 265, 280 and 315 nm standardized to the phosphorus content as follows. The calibration system was the mouse fibroblast culture L 929 for which the DNA content after nucleic acid extraction with 5 per cent TCA at 90° C has been determined according to Schneider, using the diphenylamine reaction (Burton 1956). The DNA phosphorus content was then computed from a DNA standard of known phosphorus content. It was found that the DNA P per cell averaged 2.3 $\times 10^{-12}$ g (mean of 3 determinations). Since the postmitotic (G1) DNA content of the tetraploid MFB culture is displaced on average from the mean of the culture by the factor 0.74 (Killander and Zetterberg 1965), the DNA P value of the G1 cells of the tetraploid culture was found to be 1.7 $\times 10^{-12}$ g.

The UV absorption at 265 and 280 nm given as 'total extinction' (E_{t}) comprises the specific absorption of the nucleic acids and the concomitant proteins as well as a certain part of the scattered and reflected light. The minimum and maximum limits for this unspecific light loss, recorded at 315 nm, have been discussed by Caspersson (1940, 1950) who regarded the reflected light portion as wave length independent (unspecific light loss at wave length $\lambda = E_{t,315}$) and the scattered light portion as obeying Rayleigh's law for the light scattering of infinitely small particles (unspecific light loss at wave length $\lambda = E_{t,315}\left(\frac{315}{\lambda}\right)^4$). Since in general the unspecific light loss is very small, the absorption at 265 and 280 nm is hardly affected by correction for one of these two limits. $E_{tot,265}/E_{t,280}$ is a function of the nucleic acid protein ratio. Mouse spermatozoa, for which the UV absorption of proteins at 265 nm is negligible owing to the relatively small amount, show quotients of 1.42 and 1.41 (minimal and maximal corrections, respectively, for the unspecific light loss). The corresponding values for $E_{t,265}$ are 6.20 μm^2 and 5.92 μm^2 (Killander 1966). For mouse lymphocytes with a double chromosome set the $E_{t,265}$ value is found between 12.5 μm^2 (minimal correction) and 10.9 μm^2 (maximal correction). Since the DNA content of mouse lymphocytes is half (0.48 ×) that of the G1 cells of the tetraploid MFB culture (Killander 1966), their DNA phosphorus content will be 0.82 $\times 10^{-12}$ g (0.74 according to Wiest and Heidelberger in mouse skin). Using the ratio P content : $E_{t,265}$ of mouse lymphocytes, the DNA phosphorus content has been calculated for mouse, bull and human spermatozoa as well as for human leucocytes. In contrast to mouse, bull and human spermatozoa, for which the

$E_{tot\,265}/E_{tot\,280}$ quotients are found between 1.42—1.38 (minimal maximal correction) the quotient for human leucocytes (mononuclear and polymorphonuclear cells) was found to lie between 1.14 and 1.08. This suggests an increased influence from the protein absorption. In fact the $E_{tot\,265}$ of between 16.7 and 14.1 μm^2 is far higher in relation to the values found for mouse lymphocytes and to the biochemically determined DNA phosphorus content, which is about the same (0.734 $\times$ 10^{-12} g Davidson *et al.*, 1957) as for mouse lymphocytes. The portion of the protein absorption at 265 nm for mouse fibroblast cells having an $E_{tot\,265}/E_{tot\,280}$ quotient between 1.22 and 1.16 has been calculated to lie between 25 to 18 per cent of the total $E_{tot\,265}$ (minimal and maximal corrections for unspecific light loss Killander 1966) the protein absorption in human leucocytes which have a lower extinction quotient should constitute at least the same percentage of the total $E_{tot\,265}$. After correction for 25 and 18 per cent protein absorption the $E_{tot\,265}$ for human leucocytes was found to lie between 12.5 and 11.6 μm^2 and to be in good agreement with the values obtained for mouse lymphocytes. Using these extinction values corrected for unspecific light loss and protein absorption the DNA phosphorus content of human leucocytes was calculated to between 0.81 and 0.84 $\times$ 10^{-12} g.

6. *The determination of DNA and RNA by UV absorption and by the Feulgen reaction*

The DNA content of mouse fibroblast cells was determined from the total extinction at 546 nm ($E_{tot\,546}$) after Feulgen staining and the total nucleic acid content from the total extinction at 265, 280 and 315 nm in a rapid scanning microspectrophotometer (Caspersson and Lomakka 1962, Lomakka 1965). The value for $E_{tot\,546}$ can be converted into uncorrected $E_{tot\,265}$ by multiplying by the $E_{tot\,265}/E_{tot\,546}$ quotient characteristic for a given amount of DNA, e.g. in mouse lymphocytes, which also served as the standard in the Feulgen procedure (Killander 1966). Subtracting this uncorrected $E_{tot\,265}$ for DNA from the $E_{tot\,265}$ for all nucleic acids gives an uncorrected non-DNA total extinction which is a fairly good approximation of the RNA content. The relative DNA and RNA contents can be computed by correcting the respective $E_{tot\,265}$ values for unspecific light loss and protein absorption. Since the ratio between total nucleic acid content and protein content is constant over the whole cell cycle, at least in mouse fibroblast cultures (Killander and Zetterberg 1965), the same correction can be used without major errors for all cells of the asynchronously growing mouse fibroblast culture.

Theoretical Basis of the Emission Measurement in the Microscale

A The measuring device

The set up of the measuring device used is shown in Fig 2 The energy source of the exciting radiation is a Xenon arc lamp (type XBO 450 W, Osram) which can be centered to the optical axis The exciting radiation is separated into its components by a quartz prism monochromator (Zeiss PMQ III) and focused onto the object plane by an ultraviolet transmissible condenser The radiation emitted from the irradiated object is collected by an objective with very little auto fluorescence and projected into the prism of a second quartz prism monochromator (Zeiss PMQ II) The portion of the emitted radiation selected for investigation is then focused onto the cathode of a photomultiplier

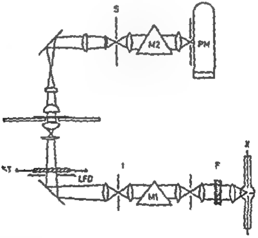


Fig 2 Plan of the measuring device for recording the emission spectra in the microscale
X = Xenon arc lamp
F = Heath protection filter
M1 = quartz prism monochromator for selecting the exciting radiation
S1 = slit of M1
LFD = light field diaphragm
ST = standard for emission measurement (uranyl glass Schott GG 17)
M2 = monochromator for analysis of the emitted radiation
S2 = slit of M2
PM = photomultiplier

The optical system is so arranged that the entrance and exit slits of both monochromators the aperture diaphragms of the condenser and the objective as well as the light arc of the Xenon lamp are projected onto one image plane and the monochromator prisms, the light field diaphragm and the object plane onto the other image plane Limitation of the measuring field is effected by the light field stop and is independent of the position of the monochromator slits Fig 3 gives a general view of the instrument in which the optical system of the UMSP I microspectrophotometer has proved convenient for object excitation

The details essential to accurate emission measurements are discussed below using this instrument as an example

1 *Centrality of the exciting light source*

For the quantitative determination of the radiation emitted by larger objects, it is necessary to have even illumination of the whole measuring field In the present experimental set up the exciting radiation at the edge of the measuring field (maximal diameter 110 μm) was not more than about 12 per cent below

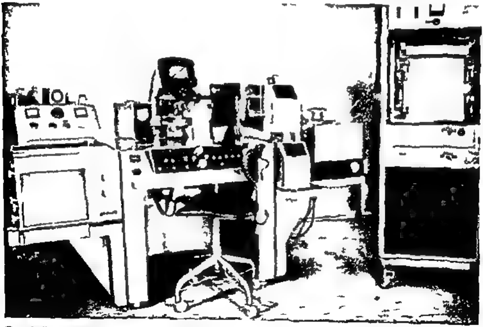


Fig 3 The recording microspectrofluorometer The two monochromators part of the housing for the xenon arc lamp and the record r for the emission spectra on the right the recorder for the absorption spectra on the left

the value at the center of the field The distribution of the exciting radiation within this measuring field was independent of the condenser magnification and the aperture when tested with a Zeiss UV Achromat 30 / N A 0 3—0 8 and a Zeiss Ultrafluar 100 / N A 0 85 Reproducible and homogeneous illumination of the measuring field was ensured by checking the centrality of the exciting light source before every series of measurements, using a small uranyl glass crystal or for the lymphocyte investigation (see Chapter 6), simply by using an AO stained lymphocyte placed in the center of the measuring field The arc of the Xenon lamp was then adjusted to give maximal emission from the uranyl glass crystal or the AO stained lymphocyte The distribution of the exciting radiation within the measuring field can be checked by moving the fluorescent test object

2 *Properties of the exciting light source*

Since the radiant energy emitted is proportional to the radiant energy absorbed the sensitivity of the emission measurement will partly depend on the radiant density or brightness of the exciting light source When measuring the very small amounts emitted by biological structures it is desirable to have an extremely sensitive measuring device and hence a high brightness of the exciting light source The brightness of a light source [candela/cm²] cannot be heightened by optical means — the image of a light source cannot radiate at a higher density than the light source itself — and is chiefly dependent on the dimensions of the light arc at constant lamp power Consequently lamps with a high radiation density have a very small arc (e g Osram HBO 100 W 2 Hg arc brightness 170 000 cd/cm², arc size 0 25 / 0 25 mm

The selection of a high radiation density light source will, apart from the arc size which could influence the homogeneity of the illuminated field, depend on its spectral characteristics (continuous or line spectrum) The properties of some typical Hg and Xenon arc lamps are given in Table I Laser light sources represent the theoretical optimum with respect to high energy output and complete monochromasy of the emitted radiation A high energy output is especially desirable when one has very high absorption of the exciting radiation and reabsorption of the emitted radiation (discussed below) this should reduce absorption and reabsorption effects since it was found that light absorption is intensity dependent at high light intensities (Condon 1964) Strict monochromasy of the exciting light source combined with a high intensity is very valuable because cutting off the exciting radiation from the emitted radiation can prove rather troublesome when broad spectral bands of the exciting

TABLE I Brightness, luminous flux, arc size and power of some typical mercury and xenon arc lamps

			Brightness (candela/cm²)	Luminous flux (lumen)	Arc size (mm)	Power (watts)
Hg arc	HBO	100 W/2	170 000	2 000	0.25 × 0.25	100
Osram	HBO	200 W/2	33 000	9 500	2.2 × 0.6	200
	HBO	500 W	30 000	28 500	4.1 × 1.1	500
Xe arc	XBO	150 W/1	15 000	3 000	2.2 × 0.5	150
Osram	XBO	450 W	35 000	13 000	2.7 × 0.9	450
	XBO	900 W	55 000	30 500	3.3 × 0.8	900
	XBO	1 600 W	65 000	60 000	4.0 × 1.4	1 600
	XBO	2 500 W	61 000	100 000	6.0 × 1.5	2 500

radiation are used to give sufficient intensity and when the absorption and emission maxima are situated close together. It is often argued that photo decomposition effects are a disadvantage of high intensity light sources, but these effects can be avoided or minimized by using registration devices with short measuring time constants (*e.g.* oscillographs) and pulse excitation.

3 *Optics*

The numerical aperture of the optical system determines the intensity of irradiation or illumination of an object situated in the object plane. The illumination J of an object of area A situated in the object plane is given by

$$J = \frac{dW}{dA} = \pi B\,(n \sin u) \qquad (1)$$

where W = radiant energy per unit time

B = radiant density or brightness of the exciting light source,

$n \sin u$ = the numerical aperture of the condenser, when u is the half size of the angular aperture.

Similarly, the illumination of the photomultiplier varies with the radiant density of the luminescent object and the square of the numerical aperture of the objective. It follows that optics with the highest numerical apertures are necessary for maximal sensitivity of the recording instrument (*e.g.* condenser Zeiss Ultrafluar 100 ×/N.A. 1.25 or 0.85, objective Zeiss Plan Apo 100 ×/N.A. 1.30 or Reichert 100 ×/N.A. 1.25 UV).

The exciting and the emitted radiation are likewise dependent on the aperture of the monochromator used for spectral analysis of the radiation. For prism monochromators the relationship is as follows

$$W = B \frac{\Delta s^2 l A}{f^2} \frac{d\lambda}{ds} \quad (2) \text{ (Kortüm, 1962)}$$

where W = radiant energy per unit time at slit width Δs

B = radiant density at the entrance slit of the monochromator per wave length unit

Δs = slit width,

l = slit height,

A = cross section of the light bundle at the prism

f = focal distance of the collimator

$\frac{d\lambda}{ds}$ = linear dispersion of the prism.

Thus the density of the radiation passing a monochromator varies with the square of the slit width. No general rules can be given as to the slit widths of monochromators these will depend on the spectral resolution required. One usually has to compromise between sufficient spectral resolution and recorded spectral intensity. For our purposes a slit width of 0.2 mm of the quartz prism monochromator giving a band width of 8.6 nm at 530 nm, was sufficient for a proper analysis of the emitted radiation while for the exciting radiation a slit width of 2.0 mm, giving a band width of 25 nm at 365 resulted in a sufficient excitation energy

Three optical arrangements are possible for excitation of an object in the microscale Fig. 5

a the exciting radiation runs in the same direction as the emitted radiation and passes through the object, realized by conventional bright field illumination longitudinal observation with the exciting radiation passing through the object

b the exciting radiation runs counter to the emitted radiation and falls upon the object realized by epi-illumination with the objective functioning simultaneously as a condenser longitudinal observation with the exciting radiation falling upon the object

c the exciting radiation passes through the object oblique to the emitted radiation realized by a dark field condenser (transversal observation)

Arrangements b and c are best for complete separation of the exciting from the emitted radiation but c involves an appreciable loss of the exciting radiation due to the central shadow of the dark field condenser, nor does it

permit simultaneous observation of the object by phase contrast which is necessary if one is to avoid redundant irradiation of the object before measurement Arrangements (a) and (b) permit high densities of the exciting radiation and simultaneous use of phase contrast observation but (a) gives a less good separation of the exciting from the emitted radiation even if prism monochromators are used, an additional filter combination on both the excitation and the emission side is required to cut out the exciting radiation In our studies we used the filter combination 325—380 nm' of the quartz prism monochromator (Zeiss PMQ III) selecting the exciting radiation to screen stray light of higher wave length than the exciting radiation (λ_{ex} = = 365 nm) and a 1 mm GG 9 (Schott) filter after the objective or even better a Reichert objective with a built in GG 9 filter situated before the front lens (which considerably reduces the autofluorescence of the objective by preventing short wave radiation from reaching lenses and glue substances *i e* the source of autofluorescence of the objective) to cut off the exciting radiation below 500 nm For technical reasons we were obliged to use the arrangement (a) although arrangement (b) is to be preferred for physical reasons (discussed below) related to the reabsorption of the emitted and the absorption of the exciting radiation The latter arrangement should give almost perfect separation of the exciting radiation from the emitted radiation if one uses a device with wave length dependent reflection (for the exciting radiation) and transmission (for the emitted radiation) (Ploem 1965)

4 *Measuring field*

The measuring field is limited by focussing the light field diaphragm onto the object plane according to Kohler s illumination principle Compared to a limitation of the measuring field by an ocular diaphragm (in the image plane) this has the advantage that only the objects to be investigated are exposed to irradiation By using slit and iris diaphragms as well as small hole diaphragms the measuring field can be varied continuously from 200 μm down to 0 3 μm Although the illumination of the measuring field is independent of the actual size of the field and varies only with the aperture of the condenser it proves to be inconstant for large variations in size as can be deduced from the variation of the fluorescence intensity of a fluorescent object when measured in measuring fields of different sizes As shown by Fig 4 increasing measuring field size by increasing the diameter of the light field diaphragm is accompanied by increasing fluorescence intensity of a fluorescent object and, hence by increasing illumination of the measuring field This increase is small for low apertures but can become considerable at high condenser apertures This

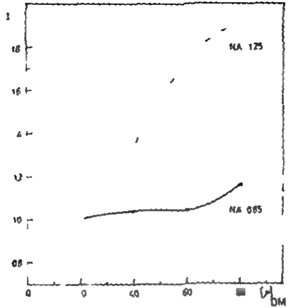


Fig 4 The intensity (I at 530 nm) of a small fluorescing object (10 μ in diameter) situated in the center of the measuring field in relation to the diameter (DM) of the measuring field

means that in cases in which the measuring field has to be varied quite often for instance to limit cell structures of different sizes (such as chromosomes) extremely high numerical apertures should be avoided when using this illumination principle in order to exclude such errors The size of the measuring field was thus in general kept constant

5 Receiver

The emitted radiation was registered with an RCA 1P 28 photomultiplier with an S5 cathode The emitted light flux was modulated by a vibrating diaphragm built into the monochromator for analysis of the emitted radiation with a frequency of 50 c p s , it was amplified by the photomultiplier using a voltage of 700 V and an A C amplifier The sensitivity limit of the A C amplifier at a signal to noise ratio of 20 1 lay at 1 mV, corresponding to an unamplified photomultiplier anode current of 0.9×10^{-10} amp Assuming a photomultiplier amplification of about 100 000 times (at 700 V) the still measurable variation of the unamplified primary cathode current corresponds to 10^{-15}—10^{-16} amp

Standardization of the measurements: constancy of the exciting radiation

A fluorescent standard made from uranyl glass (Schott GG 17) was used in order to get comparable measurements, i.e. to keep the illumination of the measuring field constant and reproducible. In addition to its high fluorescence efficiency, this standard has its emission maximum in the spectral region of the AO emission. The standard is placed between the light field diaphragm and the condenser so that the emitted radiation falls through the condenser and the specimen. The emitted radiation of the standard is measured by adjusting a free region of the specimen background. This has the advantage that the specimen can be left in place when measuring the standard emission and, hence, the intensity of the exciting radiation. Deviations in the intensity of the exciting radiation can be compensated by adjusting the slit of the first monochromator (M1). The standard gives a reproducibility of the object emission with a relative standard deviation of 3 per cent. In general, this correction for fluctuations of the exciting light intensity is very small since with appropriate air cooling the maximal deviation of the mean intensity of the xenon arc lamps used in our system (XBO 450 W) was no more than ± 5 per cent over a time interval of 30 minutes. In later experiments, a double beam arrangement was used with the standard placed in the reference light beam. Exciting light source fluctuations could then be compensated automatically by alternate registration of the intensity of the object light beam and the reference light beam with a ratio recorder.

B. Calculation of the emitted radiation

Since objects excited to emission do not always exist in enough small concentrations or layer thickness that make measurement of the emission a simple procedure, the theoretical background to the emission measurement of objects with definite concentrations and layer thicknesses will be discussed. A further complication is that the direction of both the exciting and the emitted radiation affects the measurement of the emission.

In general, the radiant energy emitted per unit time I (intensity) by a luminescent body is given by the radiant energy absorbed per unit time W multiplied by the quantum yield of fluorescence Q (light quanta emitted per light quanta absorbed):

$$I = W Q \quad (3)$$

When a parallel light bundle of wave length λ strikes a cubic body of side length a (Fig. 5 A), the radiant energy per unit time W'_{λ} falls to $W_{\lambda}(x)$ at a depth x beneath the surface according to Lambert's absorption law

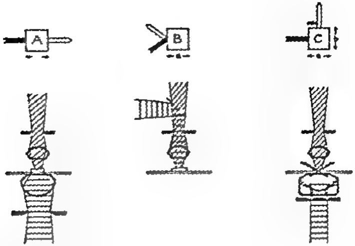


Fig 3 Three ways of measuring emission

A longitudinal observation with exciting radiation passing through the object (realized by bright field illumination)

B longitudinal observation with exciting radiation falling upon the object (realized by epi-illumination)

C transversal illumination (realized by dark field illumination) For explanation of symbols a b and x see text

$$W_\lambda(x) = W_\lambda \, e^{-K_\lambda x} \quad (4)$$

where K_λ = absorption coefficient at the absorption wave length λ

The radiant energy absorbed within a layer thickness dx is then given by

$$dW_\lambda(x) = W_\lambda \, K_\lambda e^{-K_\lambda x} \, dx \quad (5)$$

For AO with overlapping absorption and emission spectra the emitted radiation will also be absorbed according to Lambert's law unless the concentration of the luminescent compound or the layer thickness is very small For case A in Fig 3 the intensity of the emitted radiation (with wave length λ') of a layer dx at a depth x from the surface is thus given by

$$dI_{\lambda'}(x) = W_\lambda \, K_\lambda \, e^{-K_\lambda x} \, dx \, Q \quad (6)$$

while after travelling the distance $a-x$ the intensity of the emitted radiation at the opposite surface is

$$dI_{\lambda'}(x) = W_\lambda \, K_\lambda e^{-K_\lambda x} \, dx \, Q \, e^{-K_{\lambda'}(a-x)} \quad (7)$$

The sum of the emission intensities of all layer thicknesses dx is then

$$\int_0^a dI_{\lambda'}(x) = I_{\lambda'} = W_\lambda \, Q \frac{K_\lambda}{K_\lambda - K_{\lambda'}} \left[e^{-K_{\lambda'} a} - e^{-K_\lambda a}\right] \quad (8)$$

where $K_{\lambda'}$ = absorption coefficient at the emission wave length λ'

For case B (Fig 5) where the emitted radiation of a layer at a depth x from the surface has to travel the same distance x again to reach the same surface the intensity varies analogously with k_λ and K_λ

$$I_\lambda = I_{0\lambda}\, Q\frac{k_\lambda}{K_\lambda + k_\lambda}\left[1-e^{-(k_\lambda+K_\lambda)x}\right] \quad (9) \quad (cf\ \text{Forster } 1951)$$

For case C (Fig 5) where the emitted radiation travels through the body transversal to the exciting radiation and to the longitudinal side a to reach the surface the emission intensity is given by

$$I_\lambda = I_{0\lambda}\, Q\frac{F_\lambda}{k_\lambda}\left[1-e^{-K_\lambda b}\right]e^{-K_\lambda} \quad (10)$$

where x is the distance from the irradiated surface and b is the length of the body's transverse side

If however the concentration of the luminescent compound in the cubic body or the dimensions of the body itself are very small so that $k_\lambda a \ll 1$ and $K a \ll 1$, the exponential functions of I_λ may be approximated by linear functions using exponential series For cases A and B we then have

$$I_\lambda = I_{0\lambda}\, Q k_\lambda a \quad (11)$$

and I varies only with the concentration of the luminescent compound (when I is proportional to the concentration according to Beer's law) and the longitudinal dimension a of the body

For case C we have

$$I_\lambda = I_{0\lambda}\, Q k_\lambda b \quad (12)$$

and thus apart from the concentration I_λ varies only with the transverse dimension b of the body

Only under these circumstances does the intensity of the emitted radiation show a simple linear relationship to the concentration of a luminescent compound in a given volume Although such favorable conditions are attainable in most cases of AO stained biological material thanks to the small layer thicknesses the possibility of an exponential dependence of the concentration and the total amount of a luminescent substance on the measured emission intensity has to be considered when certain thicknesses are exceeded (see Chapter 6) Knowing these exponential relationships between absorption coefficients and emission intensity it is easy to correct the amount of luminescent substance underestimated by the measured luminescence intensity In this manner even emission spectra which are distorted by reabsorption of the emitted light can be corrected to give the true emission distribution This was necessary, for instance, when highly concentrated AO solutions were measured

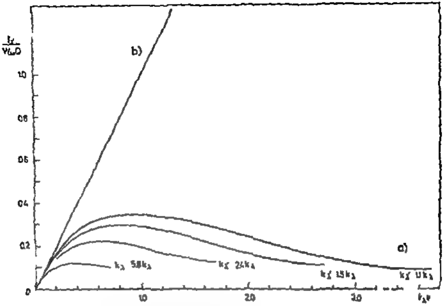


Fig 6 Graph showing the value $\frac{I_\lambda}{W_{\lambda_0}Q}$ (I_λ = radiant energy emitted per unit time (int sity) W_{λ_0} = radiant energy absorbed per unit time Q = quantum efficiency) as a fu tion of the absorption coefficient k_λ times layer thickness a

a) values for $\frac{I_\lambda}{W_{\lambda_0}Q}$ for different ratios F_λ/k_λ when $\frac{I_\lambda}{W_{\lambda_0}Q} = \frac{k_\lambda}{k_\lambda - F_\lambda}(e^{-F_\lambda a} - e^{-k_\lambda a})$

b) values for $\frac{I_\lambda}{W_{\lambda_0}Q}$ when $F_\lambda a \ll 1$ and $k_\lambda a \ll 1$ and $\frac{I}{W_{\lambda_0}Q} = k_\lambda a$

in microcuvettes (see Chapter 3) For this purpose the ratio $\frac{I_\lambda}{W_{\lambda_0}Q}$ (equati 8) was calculated for increasing values of $k_\lambda a$ and $F_\lambda a$ and arranged in a tab using an IBM 1401 electronic computer The quotient $\frac{I_\lambda}{W_{\lambda_0}Q}$ can be re from the table knowing $k_\lambda a$ and $F_\lambda a$ and extrapolated to the ideal case wh $\frac{I_\lambda}{W_{\lambda_0}Q} = k_\lambda a$ For some values of this table a graph has been made (Fig 6

As discussed above it can be an advantage to record the emitted radiati on the same side as the exciting radiation (Fig 5 case B) using epi illuminati in the microscope if the absorption of the body excited to luminescence very large and $k_\lambda a \gg 1$ Equation (9) then takes the following form

$$I = W_{\lambda_0} Q \frac{k_\lambda}{F_\lambda + k_\lambda} \quad (13)$$

The measured luminescence intensity is then independent of the thickness a of the body, and the spectral distribution of the emitted radiation shows very little distortion due to reabsorption

CHAPTER 3

The Binding of Acridine Orange to Nucleic Acids

Influence of the nucleic acids' secondary structure

The mechanism by which acridine orange (AO) is bound to biological structures was first interpreted by Strugger (1940). Influenced by the work of Scheibe (1939) on the reversible polymerization of pseudoisocyanine, he assumed similar relations in the case of acridine orange and explained the metachromatic change of the fluorescence in dead cells in terms of an increasing concentration of the dye ('concentration effect"). The physical chemistry of the absorption and emission of AO solutions of different concentration has been investigated in detail by Zanker (1952 a, 1952 b, 1959), confirming Strugger's assumption.

Stimulated by the pioneer work of Michaelis (1947) on the interaction of nucleic acids and basic dye stuffs, Peacocke and Skerrett (1956), followed by Steiner and Beers (1957) and Bradley and Wolf (1959), investigated the binding of amino acridines to nucleic acids, which constitute the reactive groups of Strugger's concentration effect. Further contributions in this field have been made by Ranadive and Korgaonkar (1960), Lerman (1961, 1963, 1964), Boyle *et al.* (1962), Weill and Calvin (1963), Borisova and Tumerman (1964, 1965), Tubbs *et al.* (1964) and Gersch and Jordan (1965).

Peacocke and Skerrett, studying the binding strength of proflavine to deoxyribonucleic acids, observed the existence of two types of bindings: a strong one at low dye binding site ratios due to a direct combination of single proflavine cations with binding sites on the DNA, and a very weak one due to a combination of proflavine aggregates with nucleic acids or to a binding of free proflavine to proflavine already bound. Beers *et al.* (1958) and Steiner and Beers (1959) investigated the spectral changes of dilute AO solutions after the step wise addition of different nucleic acids and synthetic nucleotide polymers and attained results comparable to those of Michaelis concerning the influence of nucleic acids on the absorption characteristics of toluidine blue. This investigation showed that the step wise addition of nucleic acids or other polyanions to monomeric AO solutions leads to a decrease of the α band of the monomeric AO absorption spectrum at 492 nm with a simultaneous increase of the β band situated at 464 nm, which attains maximal intensity at a polymer dye ratio of 1 : 1 (Complex I of Steiner and Beers). Certain polyanions, as shown by Appel and Zanker (1958), give rise to a pronounced

third band at 450 nm (γ band). The further addition of polyanions causes a reappearance and steady increase of the α band with a bathochromic shift to 502 nm (Complex II of Steiner and Beers). The amount that has to be added in order to cause a reappearance of the shifted α band varies with the type of polyanion used (Bradley and Wolf 1959, Bradley and Felsenfeld 1959, Bradley, 1961, Stone and Bradley 1961). Using these quantitative spectral differences, the terms "stacking tendency" and "stacking coefficient" have been defined by these authors as a probability measure for the formation of dye aggregates on the binding sites of different polyanions. Thus the stacking coefficient for DNA is 1.25, for RNA 3.3, for Poly U 109 and for heparin 787. Whereas Steiner and Beers explain the formation of Complex I by the binding of AO to 6-amino groups and Complex II by the binding of AO to terminal phosphate groups, Bradley relates these spectral changes to different degrees of dye aggregation in the binding of the cationic AO to the anionic phosphate groups. This view is supported by Zanker's investigation of AO in aqueous solutions (1952 a), in ethanol ether mixtures and in ethanol (1952 b, 1959): the α, β and γ bands were interpreted as 0—0, 0—1 and 0—2 bands of the first electron transition that are due to association of the AO cations in solvents with high dielectric constants that favor the efficiency of the London-van der Waals dispersion forces.

A. Spectral analysis of nucleic acid-AO complexes

The results of Steiner and Beers and those of Bradley *et al.* are not directly comparable with the findings from the AO staining of biological material since the former were derived from a mixture of free AO and AO bound to nucleic acids. Since AO stained biological material represents a system in which AO is found only in its bound state, the present aim was to investigate pure nucleic acid-AO complexes which are quite insoluble in the concentrations found in a cell, *i.e.* they form precipitates. This form of nucleic acid-AO complex could be imitated by the microdroplet technique, by which nucleic acids sprayed on glass slides form droplets of about cell size and smaller; these are then stained by AO. A considerable problem with such precipitates is the high reabsorption of the emitted light in the nucleic acid-AO complex itself. This effect, which calls for rather time-consuming correction, was avoided by using microdroplets with layer thicknesses of 0.1—0.7 μm; this kept the amount of reabsorbed radiation below 2 per cent of the total emitted radiation.

The nucleic acid microdroplets were stained in 10^{-4} M AO (Citric acid-Na_2HPO_4 buffer, pH 6.5, ionic strength 0.24) in a thermostatic staining bath (Fig. 1), which insures reproducible diffusion of the dye to the nucleic acid

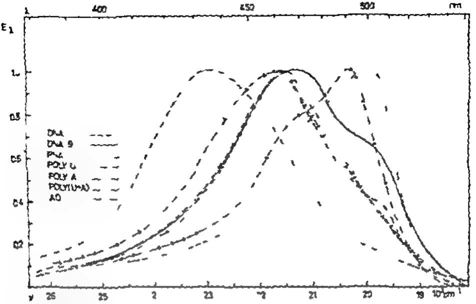


Fig 7 Absorption spectra of different nucleic acid AO complexes and a 10⁻⁵ M AO solution Maximal extinction value (E) = 1 0 $\bar{\nu}$ = wave number λ = wave length Citric acid Na_2HPO_4 buffer pH = 6 5 μ = 0 24

droplets in the dye bath as well as of the unbound dye from the nucleic acid AO complex into pure buffer solution, their absorption and emission spectra were recorded by the microspectrofluorometer described in Chapter 2 An absorption maximum of the AO complex of native calf thymus DNA is found at 20 000 cm^{-1} and a shoulder at 21 000 cm^{-1} (Fig 7) Heating the native DNA in ion free water at 90 C for 30 minutes followed by immediate cooling in an ice bath — a process which is known to lead to the formation of single stranded chain structures (Doty *et al*, 1960, Marmur *et al* 1963) — reverses the situation *i e* there is a maximum at 21 300 cm^{-1} and a shoulder at 20 000 cm^{-1} A similar situation occurs with the yeast ribosome RNA AO complex, with a maximum at 21 600 cm^{-1} and a much less pronounced shoulder at 20 000 Similarly the Poly A AO complex has a maximum at 21 600 cm^{-1} and an additional shoulder at about 23 000 cm^{-1} It is known that, at the high ionic strength in question (0 24), these two nucleic acids behave as flexible coils with intermittent helical regions of varying size (Fresco and Doty 1957, Doty *et al* 1959 Fresco *et al* 1960 Boedtker 1960 Rich *et al* 1961, Spirin, 1963 1964) In the case of the Poly U AO complex the broad shoulder of the Poly A AO complex at 23 000 cm^{-1} develops to the absorption maximum

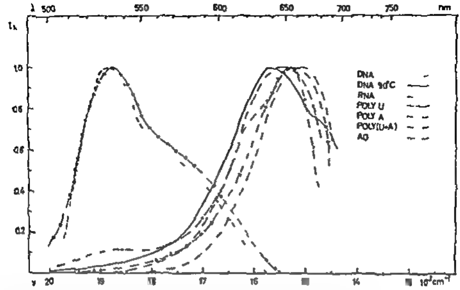


Fig 8 Emission spectra of different nucleic acid AO complexes and a 10 ⁴M AO solution Maximal intensity value $(I) = 1.0$ Citric acid Na_2HPO_4 buffer pH = 6.5 $\mu = 0.24$ Correction for spectral changes in photomultiplier sensitivity and in the transmission of the optical system Excitation at 365 nm

while a secondary shoulder develops at 21 600 cm⁻¹ Poly U is the sole example of a polynucleotide for which no organized fine structure has been demonstrated (Rich 1957 Steiner and Beers, 1961)

To test this apparent correlation between decreasing order of the nucleic acid structure and the shift toward blue of the absorption maxima of the nucleic acid AO complex an equimolar mixture of Poly U and Poly A was investigated This mixture of polynucleotides is known to show a high degree of ordered fine structure in its X ray diffraction pattern which is reminiscent of that produced by natural DNA (Rich and Davies 1956 Rich, 1957) It was found that the absorption spectra of the Poly (U + A) AO complex had almost completely lost the broad absorption band at 23 000 cm⁻¹ displayed by the two polynucleotides separately while a distinct side peak around 20 000 cm⁻¹ reappeared in addition to an absorption maximum at 21 600 cm⁻¹ This result agrees with the swinglike behavior of the different absorption bands of the nucleic acid AO complexes the long wave absorption bands are emphasized when AO binds to nucleic acid structures with a high structural organization while the short wave absorption bands predominate when AO binds to nucleic acids with a poor chain organization

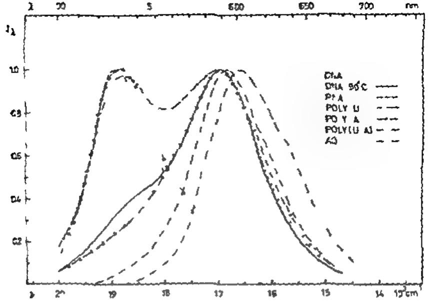


Fig 9 Emission spectra of nucleic acid AO complexes No correction for spectral changes in photomultiplier sensitivity and in the transmission of the optical system.

Similar results have been obtained from the emission spectra of these nucleic acid AO complexes though the picture is exactly the reverse of that for the absorption spectra (Fig 8) Thus, the native DNA AO complex shows an emission maximum at 18 800 cm⁻¹ and a broad shoulder around 17 200 cm⁻¹ After denaturation of DNA the emission maximum shifts into the red region at 15 700 cm⁻¹ while the emission at 18 800 cm⁻¹ characteristic of the native DNA has completely disappeared The RNA AO emission is rather similar to that of denatured DNA although the emission peak is shifted slightly to 15 400 cm⁻¹ The Poly A AO complex shows an additional shift of the emission maximum to about 15 300 cm⁻¹ while the emission maximum of the Poly U AO complex is situated at 15 000 cm⁻¹ In the Poly (U + A) AO complex the emission at 18 800 cm⁻¹ characteristic of the native DNA AO complex is again recognizable

Summarizing the properties of the emission spectra of nucleic acid AO complexes when the structural order of the acids is lost there is a decrease of the short wave emission bands with a successive shift of the emission maximum toward the red and vice versa Essentially the same behavior is found when no correction is made for the wave length dependent photomultiplier sensitivity and the transmission of the optical system however the emission bands in the green region are more accentuated than in the corrected spectra and the

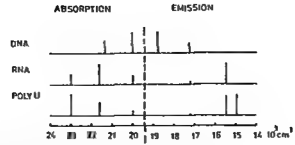


Fig 10 Schematic representation of the position and the relative intensities of absorption and emission bands of DNA AO RNA AO and Poly U AO complexes

bulk of the emission maxima found in these around 15 500—15 000 cm^{-1} is displaced to 17 000—16 500 cm^{-1} because the sensitivity of the photomultiplier used (RCA 1P 28) is much higher in the green region than in the red (Fig 9)

The centres of gravity of the absorption and emission bands constituting the different main and side peaks of the nucleic acid AO spectra were plotted on a scale that gives the energy differences in terms of the wave number (Fig 10) the exact position and relative intensity of the superimposed bands have been determined by a graphic isolation procedure (Kortum 1962) It will be seen that there is an obvious symmetry in the position and relative intensity of the absorption and emission bands especially in the case of the DNA AO complex This type of mirror symmetry, first described by Nichols and Merrit (1910) and later also by Lewshin (1931), is in good agreement with the results of Zanker (1952 b 1959) concerning the absorption and emission bands of AO solutions As in the case of AO solutions of different concentrations three main bands at 20 000 21 600 and 23 000 cm^{-1} can be isolated in the absorption spectra which are matched by bands in the emission spectra at 18 800 17 200 and 15 700—15 400 cm^{-1} The absorption bands in AO solutions have been interpreted by Zanker as being caused by the transition from the zero vibrational level of the ground state to the zero the first and the second vibrational level of the excited electronic state (0—0 0—1 and 0—2 transition bands), and the emission bands as the transitions from the zero vibrational level of the excited electronic state to the zero the first and the second vibrational level of the ground state The 0—0 bands were related to the monomer AO cation in dilute solutions the 0—1 and 0—2 bands to dimer and tetramer associates in concentrated solutions The successive prevalence of the short wave absorption bands and the long wave emission bands with increasing AO concentration as found by Zanker can also be demonstrated when AO binds to nucleic acids suggesting an increased AO concentration on

nucleic acid chains with a decreasing order of their chain structure The increased probability of transitions from the ground state to higher vibrational levels of the excited electronic state (expressed by intensified short wave absorption bands) is accompanied by an increased probability of transition from the excited electronic state to higher vibrational levels of the ground state (as indicated by intensified long wave emission bands) and can be explained (Zanker 1952 b 1959) in terms of the Franck Condon principle (Forster, 1951 Pringsheim, 1949) Thus, the altered transition probabilities are caused by a shift in the equilibrium of the excited electronic state that is due to an increased contact of dye molecules

This symmetry in the intensity and position of absorption and emission bands, which is found in the nucleic acid AO complexes up to *23 000* cm^{-1} on the absorption side and up to 15,700—15 400 cm^{-1} on the emission side, can be observed for the DNA AO and the RNP AO complexes (Fig 20 Chapter 4) For the RNA AO complex however, this symmetry is somewhat disturbed and in the Poly U AO complex even more so, since an additional emission band (or bands) appears at 15 000 cm^{-1} with no distinct equivalent on the absorption side The same behavior has been displayed by AO solutions of increasing concentration indicating that when bound to nucleic acids, AO shows the same molecular configurations as in solution The appearance of additional emission bands without a counterpart on the absorption side can hardly be explained by a direct transition from the excited state to the ground state and points to an emission process of a different nature

The occurrence of such long wave emission bands indicates an additional loss of vibrational energy This can arise if the emission process involves a radiationless transition *from the excited state over an intermediate metastable* state to the ground state Forster (1951) has explained the existence of such a metastable state as being in theory a result of the association of flat and symmetric dye molecules *e g* thionine or acridine orange According to this theory two associated dye molecules each having an electronic oscillator vibrating along the molecule s long axis are first elevated to an excited state with both electronic oscillators vibrating in phase, after which the double molecule passes to an intermediate metastable state of lower energy with the electronic oscillators vibrating out of phase From this state the associated molecule reaches the ground state by a radiationless process or by a prolonged long wave emission since the transition from the metastable to the ground state has a low probability (forbidden transition) the metastable state is maintained for a certain period and gives raise to an afterglow of the molecule This type of emission process, characterized by a moderately prolonged lifetime (5×10^{-4}—10^{-2} sec) seems to be applicable

also for the emission of nucleic acid AO complexes at long wave lengths Preliminary measurements have shown that the emission of the RNA AO and Poly U AO complexes at all wavelengths have a lifetime of less than 10^{-8} seconds, a phosphorescence process of long duration at room temperature can therefore be excluded More recently Borisova and Tumerman (1964 1965) found that the denatured DNA AO complex in solution had a lifetime of 25×10^{-9} seconds for the emission at 640 nm which was five times longer than the lifetime of the native DNA AO complex Such a result favors the interpretation of this emission process in terms of Forster s theory However, since these measurements were apparently made at room temperature the existence of an emission process of long duration at low temperature as found in AO solutions (Zanker 1952 b) cannot be excluded In such a case the red emission would be better explained by Lewis Kasha s triplet singlet transition theory (Lewis and Kasha, 1944) The excited molecule passes from the excited state where it has electron pairs with anti parallel spins (↑↓, excited singlet state) to a metastable state with parallel electron spins (↑↑ triplet state) The transition to the ground state (singlet state) is very improbable and any emission will show greatly extended lifetime

The existence of such metastable states can be demonstrated from the degree of polarization of the emitted light when excited by linear polarized light since the degree of polarization is a function of the angle between the absorp tion and the emission oscillators for which Perrin (1929) has given the following equation

$$p = \frac{3\cos^2\alpha - 1}{\cos^2\alpha + 3} \qquad (14)$$

where p = degree of polarization given as $\frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$ ($I_{\parallel}$ $I_{\perp}$ components of the emitted light with their vibrational planes parallel and transverse to the direction of the exciting polarized light),

α = angle between the absorption and emission oscillators

Clearly with parallel absorption and emission oscillators ($\alpha = 0$), p is 0 5 and positive, p is negative when the angle between the oscillators is greater than 55 and becomes — 0 3 at maximal distortion (90°) Since an emission process passing from an excited state of higher frequency via a metastable state of lower frequency to the ground state leads to distortion of the emission oscillator against the absorption oscillator such a process can be assumed in the case of low degrees of fluorescence polarization This is particularly so if negative values are observed as they are in the measurement of the fluorescence polarization spectra of RNA AO and Poly U AO complexes excited whit

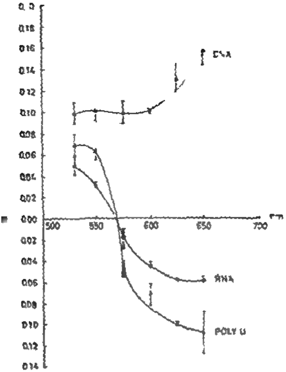


Fig 11 Fluorescence polarization spectra of DNA AO RNA AO and Poly U AO complexes pH = 6 5 μ = 0 24 Excitation by polarized light at 365 nm

linear polarized light of wave length 365 nm (Fig 11) $p = -0.06$ for RNA AO and $p = -0.11$ for Poly U AO at 15 400 cm^{-1} (650 nm) On the other hand the native DNA AO complex gives a corresponding p of $+0.16$ which is in good agreement with Weill and Calvin s (1963) $p = +0.175$ for the DNA AO complex at a polymer dye ratio of 6 1 The angle between the absorption and emission oscillators is much smaller in the case of this complex and the existence of a metastable state as in the RNA AO and Poly U AO complexes seems less likely Moreover p was determined at room temperature at which the Brownian rotational movement decreases the actual degree of polarization positive as well as negative This means that the actual degree of polarization of the DNA AO as well as of the RNA AO and Poly U AO complexes may be much greater when measured at low temperature or in highly viscous media

Summarizing the results obtained from the study of the absorption emission and fluorescence polarization spectra it can be said that AO binds to nucleic acids with a high structural order (helix) in a monomer molecular form as indicated by the prevalence of the absorption bands at 20 000 cm^{-1} and the

emission bands at 18 000 cm⁻¹ constituting an 0—0 transition from the ground state to the excited electronic state and *vice versa* This is also indicated by the fact that monomer AO solutions display the same spectral distribution (see Figs 7 and 8) apart from a minor shift of the absorption band to a shorter wave length and of the emission band to a longer wave length characteristic of unbound AO molecules (Bradley and Wolf 1959 Stone and Bradley 1961 Weill and Calvin 1963) On the other hand AO binds to nucleic acids with a low structural order (flexible and random coils) in an associated molecular form as indicated by the heightening of the absorption bands at 21 600 cm⁻¹ and 23 000 cm⁻¹ and of the emission bands around 17 200 cm⁻¹ and between 15 700 cm⁻¹ and 15 000 cm⁻¹ For the emission process two mechanisms can be assumed The first one valid for all nucleic acid AO complexes is due to direct transitions from the excited electronic-state to different vibrational levels of the ground state the probability of each transition being determined by the Franck Condon principle The second mechanism found only with nucleic acid AO complexes with low-order nucleic acid chains is due to intermediate metastable states during the emission process causing negative degrees of polarization and prolonged long wave emission

B Quantitative evaluation of emission spectra as a method for determining the degree of order of nucleic acid chains

The apparent correlation of the decrease of short wave emission bands and the appearance of long wave emission bands to the decrease in the structural order of nucleic acid chains when binding AO prompted a search for a quantitative relation between the degree of chain ordering and the emission properties of the corresponding AO complex The two emission bands chosen for this investigation are common for all nucleic acid AO complexes and can be attributed to the same emission mechanisms Thus the emission (fluorescence) intensities selected at wave length 18 870 cm⁻¹ (530 nm denoted as F_{530}) and at wave length 16 950 cm⁻¹ (590 nm denoted as F_{590}) may be interpreted as direct transitions from the excited state to the ground state obeying the Franck Condon principle At the same time these two emission intensities caused by AO bound in monomer molecular form (F_{530}) and in associated molecular form (F_{590}) constitute the emission peaks of the uncorrected emission spectra of high-ordered nucleic acid AO complexes (*e g* DNA AO see Fig 9) and of low-ordered nucleic acid AO complexes (denatured DNA AO, RNA AO Poly U AO) thus a high measuring sensitivity for both wave lengths in the measuring system used is ensured

The ratio between the two fluorescence intensities at 590 nm and at 530 nm

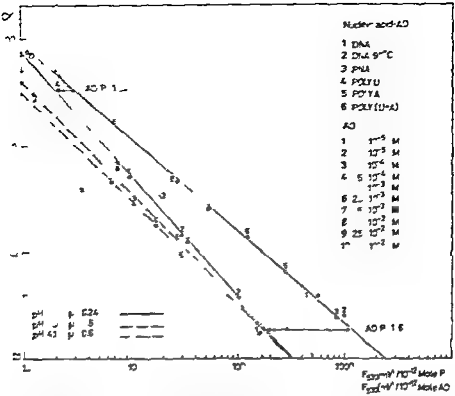


Fig. 12. Graph showing α (the ratio of the fluorescence intensities at 590 nm and 530 nm F_{590}/F_{530}) for the binding of acridine orange to nucleic acids with different structural order of their chains as a function of the fluorescence intensity at 530 nm per 10^{-12} mole nucleic acid phosphorus (F_{530} [mV] 10^{-12} mole P). pH = 6.0, μ = 0.24 (——— ●); pH = 6.0, μ = 0.6 (— — — ▲); pH = 4.1, μ = 0.6 (—·— — ■). Regression lines and upper 95 per cent confidence interval for pH 6.0, μ = 0.24; lower 95 per cent confidence interval for pH 4.1, μ = 0.6. α of acridine orange solutions of different dye concentrations as a function of the fluorescence intensity at 530 nm per 10^{-12} mole acridine orange (F_{530}[mV]/10^{-12} mole AO) pH = 6.0, μ = 0.24 ——— ○).

F_{590}/F_{530} denoted as α is shown below to be a measure of the degree of dye aggregation on the nucleic acid chain. For all the nucleic acid AO complexes investigated, this ratio α was compared on a basis common to all nucleic acids, namely the phosphorus content. For this purpose the α of all nucleic acid AO complexes was plotted against the fluorescence intensity of the monomer dye molecules at 530 nm emitted per 10^{-12} mole phosphorus ($F_{530}/10^{-12}$ mole P, Fig. 12) for different pH and ionic strengths. As will be seen from Fig. 12, the values for α display a functional dependence on the monomer dye intensity emitted per 10^{-12} mole nucleic acid phosphorus; this function,

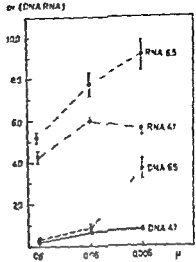


Fig 13 Relation between α of DNA AO and RNA AO complexes and the ionic strength (μ) at different H ion concentrations (pH = 4.1 and 6.5)

a rational is common to all nucleic acid AO complexes stained in the same pH and ionic strength For pH 6.5 $\mu = 0.24$ for instance it takes the form

$$\alpha = 75.1\ (F_{530}/10^{-12}\ \text{mole P})^{-1.15} \quad (15),$$

for pH 4.1, $\mu = 0.6$

$$\alpha = 30.1\ (F_{530}/10^{-12}\ \text{mole P})^{-0.69} \quad (16)$$

The different slopes of the functions indicate a reduced increase in the size of α with decreasing structural order when nucleic acids are stained at ionic strengths above 0.24 and pH below 6.5 In the case of higher ionic strength this lower tendency of AO to aggregate on the nucleic acid chain is explainable by the interaction of ions with equal charge (Na^+) in the case of lower pH it may be due to an increased positive charge of the AO cation leading to an increased molecular repulsion In addition the degree of structural order of nucleic acids is in general decreased at low ionic strength (helix-coil transition) This dependence of α on the ionic strength and the pH is seen even better when the range of the ionic strength is more extended (Fig 13)

Since the fluorescence properties of all nucleic acid AO complexes having big differences in their degree of chain order obey, with minor differences the same function, this suggests a functional dependence between the size of α and the monomer fluorescence intensity of AO bound per 10^{-12} mole P on the one hand, and the degree of chain order on the other (a decreasing chain order being connected with an increase in α and a decrease in $F_{530}/10^{-12}$ mole P) The extreme values for α are thus found at the Poly U AO complex

(single stranded random coil) and at the DNA AO complex (double stranded helix)

If a nucleic acid of unknown structural order may be regarded as being composed of varying fractions of pure double stranded helix (DNA) and pure single stranded random coil (Poly U) structures the fluorescence intensity of its AO complex can be given as the sum of the intensities characteristic for each constituent The fluorescence intensity at 530 nm is then denoted as

$$F_{530} = D f^{D}_{530} + S f^{S}_{530} \quad (17)$$

where F_{530} = fluorescence intensity (not corrected for spectral dependent photomultiplier sensitivity see methods) at 530 nm given in mV (amplified photomultiplier signal)

D S = the amount of double stranded (helix) and single stranded (random coil) regions given in 10^{-1} mole P

f^{D}_{530}, f^{S}_{530} = fluorescence coefficients , fluorescence intensity at 530 nm per 10^{-12} mole P ($F_{530}/10^{-12}$ mole P) emitted by the double stranded helix (DNA) AO and by the single stranded random coil (Poly U) AO complex (see Fig 12 and 14)

The corresponding expression for the second wave length 590 nm is

$$F_{590} = D f^{D}_{590} + S f^{S}_{590} \quad (18)$$

That the fluorescence coefficient is a real constant for the single stranded AO complex as well as for the double stranded AO complex is demonstrated by the linear relationship between the fluorescence intensity at the wave lengths 530 and 590 nm and the phosphorus content of Poly U AO complexes and DNA AO complexes (Fig 14)

Simplifying both equations (17) and (18), the ratio S/D is given by

$$S/D = \frac{a - a_D}{a_S - a} C \quad (19)$$

where a = the ratio F_{590}/F_{530} for the nucleic acid fraction in question

$a_D = f^{D}_{590}/f^{D}_{530}$ for the pure double strand AO complex

$a_S = f^{S}_{590}/f^{S}_{530}$ for the pure single strand AO complex,

$C = f^{D}_{530}/f^{S}_{530}$

The validity of this equation has been checked at pH 4 1 for different mixtures of DNA and RNA (the latter being regarded as almost completely single stranded) for which there is a linear relationship between the ratio RNA/DNA and the expression $\frac{a - a_D}{a_S - a}$ (Fig 23 see Chapter 4)

Applying equation (19) to the nucleic acids investigated gives the following relative amounts of double stranded helix (ordered) and single stranded

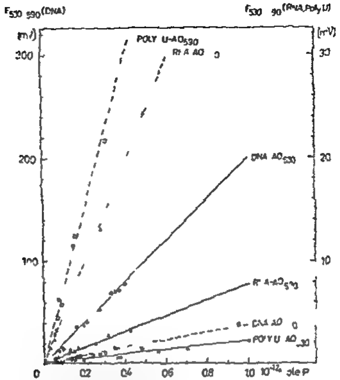


Fig 14 Graph showing the stoichiometric relation between the fluorescence intensity at wave length 530 nm and 590 nm (F (mV)) and the phosphorus content of DNA AO RNA AO and Poly U AO complexes pH = 6.5 μ = 0.24

random coil (disordered) regions at pH 6.5, ionic strength 0.24 DNA is assumed to be completely double stranded, $a_{\Pi} = 0.187$, and Poly U completely single stranded $a_s = 33.1$ $C = 90.0$ (Table II)

The amount of double stranded helix regions expressed in terms of nucleic acid phosphorus decreased to 10 per cent of the initial value when native DNA was heated (see Table II) The corresponding figures for ribosomal yeast RNA and the alkaline form of Poly A were only 5 and 8 per cent respectively For the equimolar Poly A + Poly U complex the figure was about 30 per cent

The amount of helix regions calculated for ribosomal RNA is smaller than that estimated for ribosomal RNA in solution on the basis of UV hypochromicity and optical rotation (Doty *et al* 1959 Fresco *et al* 1961) Apart from the fact that this RNA fraction with an $\varepsilon^P_{260} = 9\,750$ must be regarded as a rather unfolded random coil (Cox and Littauer 1962) there may be another reason for the low content of helix regions The helix regions in ribosomal RNA are flexible reversible and highly dependent on the environment (ionic

When binding to the single stranded random coil, AO is bound by almost every nucleotide unit (i.e. its PO_4 group), resulting in a dye-dye interaction that produces an emission spectrum with prevailing bands between 15 400 cm^{-1} and 15 000 cm^{-1} (650—665 nm), which is equivalent to the red emission associated with cytoplasmic RNA in AO stained cells and to the "Complex I" of Steiner and Beers. These two models are very similar to those derived by Bradley and Wolf (1959) from the varying ability of nucleic acids to stack acridine orange. Moreover, the quotient, which is determined by the AO : P ratio of a nucleic acid AO complex, is comparable to Bradley's stacking coefficient "k", which is a measure for the tendency of dye molecules to aggregate when binding to polyanions.

Summary

The absorption and emission peaks of AO bound to nucleic acids of different chain order (helix, random coil) constituting the center of gravity of different absorption and emission bands are comparable to those found with AO solutions of different concentrations. They are found at 20,000 cm^{-1} (500 nm), 21,600 cm^{-1} (463 nm) and 23,000 cm^{-1} (435 nm) in the absorption spectrum and at 18 800 cm^{-1} (532 nm), around 17 200 cm^{-1} (580 nm) and between 15 700 and 15,000 cm^{-1} (635—665 nm) in the emission spectrum. The changes in both the absorption and the emission spectra can be explained by a different aggregation of AO molecules on nucleic acid chains with varying degrees of structural order.

The absorption band at 500 nm 20 000 cm^{-1} (500 nm) and the emission band at 18 800 cm^{-1} (532 nm) predominate when AO binds to nucleic acids of the helix type, indicating that the AO is bound in a monomer molecular form. The AO : nucleic acid phosphorus ratio has been found to be 1 : 6 showing that approximately one AO molecule is bound per three base pairs. Conversely when AO binds to nucleic acids of the random coil type (Poly U) the absorption band at 23 000 cm^{-1} (435 nm) and the emission bands between 15 400 and 15 000 cm^{-1} (650—665 nm) predominate, indicating that the AO is bound in an associated molecular form. The AO : P ratio is 1 : 1.5 suggesting that almost every base pair binds one AO molecule. Nucleic acids containing both random coil and helix regions (ribosomal RNA, Poly A) have their maximal intensity at absorption and emission bands situated between those characteristic for helix AO and random coil AO complexes.

While the emission of AO bound to the helix chains in a monomer molecular form is due to a direct transition from the excited electronic state to the ground state (fluorescence), the emission of AO bound to random coil chains in an associated form is caused by a transition from the excited electronic state over

an intermediate metastable state, causing negative polarized emission and a prolongation of the emission process (phosphorescence)

A method has been proposed for determining the relative amounts of helix and random coil regions in nucleic acids after complex formation with AO using the ratio between the fluorescence intensities at 590 nm (16,9ɔ0 cm^{-1}) and 530 nm (18 870 cm^{-1}) this ratio, α is a function of the AO P ratio and increases with decreasing stuctural organization of the nucleic acids

The Binding of Acridine Orange to Nucleoproteins

Influence of the protein component

Since the binding of the protein fraction of the nucleoprotein complex (Zubay and Doty, 1959 Bayley *et al* 1962) as well as that of AO to the nucleic acid must be regarded as being primarily effected by electrostatic forces, it must be assumed that the protein component exerts a considerable influence on the binding of AO onto nucleoproteins

For this investigation two nucleoproteins with well known chemical and physical properties were used, namely deoxyribonucleoprotein (DNP), isolated from calf thymus, and ribonucleoprotein (*ribosomes*) isolated from rat liver

Both nucleoproteins consist in a purified state of one part nucleic acid and one part protein (DNA/DNP = 0 47 Zubay and Doty, 1959 RNA/RNP = 0 41—0 46 Hamilton and Peterman, 1959, Kirsch *et al*, 1960) but are distinguished by their primary, secondary and tertiary structure

From the optical and hydrodynamic properties of DNP (Zubay and Doty 1959 Murray and Peacocke, 1962, Bayley *et al*, 1962, Giannoni and Peacocke, 1963 Lee *et al*, 1963 Giannoni *et al*, 1963) as well as from the X ray diffraction pattern (Zubay and Wilkins, 1962, 1964) it must be concluded that DNA shows the same highly organized double helix structure in combination with histones as in the isolated state On the basis of the X ray diffraction patterns Zubay (1964) surmised that the histone molecules lie parallel to the large groove of the DNA with their long axis at an angle of 60 to the long axis of the DNA connecting several DNA molecules together

Ribosome particles too, display a certain structural organization of the RNA (Rich and Watson, 1954 a b Hall and Doty, 1959, Schlessinger, 1960, Zubay and Wilkins 1960 Klug *et al*, 1961, Langridge and Holmes, 1962, Spirin 1963 1964) This indicates the presence of helical regions, as found in isolated ribosomal RNA (Doty *et al*, 1959 Fresco *et al*, 1960, Cox and Littauer, 1962 Cox 1963) In Spirin's (1964) hypothetical model the ribonucleic acid chain, which shows a varying degree of helix regions impregnated by the concomitant protein is either folded into a flat rectangle (the 30 S particle) or into a pentagonal prism (the 50 *S* particle)

The main difference between the histones and the ribosomal proteins is that the amount of acidic amino acids (aspartic acid, glutamic acid) is larger in

the latter while the relative amount of basic amino acids (arginine, histidine and lysine) is about the same in both (24—26 %) Thus the ratio between basic and acidic amino acids is for histones from calf thymus 1 77—1 98 (Crampton *et al*, 1957, Phillips 1962) and for ribosomal protein from rat liver 1 33—1 37 (Cohn and Simson 1963)

The nucleoproteins may be distinguished from their nucleic acids by the additional anionic and cationic groups which belong to the protein part The cationic protein groups compete with the equally charged dye for the anionic phosphate groups of the nucleic acid the anionic protein groups like the phosphate groups bind AO Since the ratio between anionic and cationic charged groups is also related to the H ion concentration the binding of AO to nucleoproteins should be influenced to a greater extent by the environmental conditions than is the case when AO binds to nucleic acids

A Spectral properties of the nucleoprotein AO complexes

The net charge of the amphoteric proteins can be changed in two ways (a) by varying the pH, which influences the dissociation of the acid and basic groups of the protein molecule (b) by blocking the basic or acid groups rendering them no longer dissociable, or by removing them Blocking the positively charged amino groups acts in the same way as shifting the pH to the neutral or basic side *i e* it increases the negative charge of the protein molecule consequently this should result in a loosening of the protein fraction from the anionic nucleic acid in the nucleoprotein complex, since both partners interact by electrostatic forces A predominance of the basic groups — as caused by a repressed dissociation of the acid groups at low pH—should lead to an increased binding of the protein to the nucleic acid

The same effect would of course result from a change in the net charge of the protein molecule due to a variation in the composition of amino acids (especially the basic or acidic amino acids) this is known to occur in nature (histone protamine transition)

To test this line of reasoning the binding of AO to DNP and RNP was investigated at two H ion concentrations namely the neutral region and at pH 4 which is the maximum at which the hydrogen bonds in highly organized nucleic acids are not irreversibly destroyed (Jordan 1955, Cox and Peacocke 1956 1957 Cavalieri and Rosenberg 1957) The basic amino groups were blocked with acetic acid anhydride this compound as well as acetyl chloride is used in organic chemistry to introduce the acetyl groups into hydroxyl compounds or into amines (Schotten Baumann reaction) in combination with alkali or pyridine to neutralize the acetic acid liberated simultaneously These

reactions have been successfully transferred to histochemistry and used to block amino groups (Monne and Slautterback, 1950, Burstone 1955) and glycol groups (McManus and Cason 1950) Other methods are the use of formaldehyde, which reacts in a similar way to acetic anhydride with amino groups and nitrous acid or chloramine T, which remove the amino groups by oxidative deamination these, however, have proved difficult to control or else they require an acid pH This is not the case with acetic acid anhydride The blockage of amino groups by acetic acid anhydride, which primarily affects the amino groups that do not constitute the peptide bond, *i e* the amino group of lysine and the guanidino group of arginine, is achieved by the reaction

$$NH_2\ R\ COOH + (CH_3CO)_2O \rightarrow CH_3CO\ NH\ R\ COOH + CH_3COOH$$

The nucleoproteins were sprayed onto glass slides in the same way as the nucleic acids to obtain microdroplets like the nucleic acids, they were stained with AO in citrate phosphate buffers of high ionic strength partly to attain a rapid diffusion equilibrium and partly to avoid an irreversible denaturation of nucleic acids with ordered structure, which is possible with low ionic strengths even at pH 4 (Cavalieri and Rosenberg, 1957) The nucleoprotein droplets were fixed in a 1 1 mixture of ethanol acetone for 30 minutes before being stained in an excess of AO they were then transferred to pure buffer solution to diffuse away any dye that was not firmly bound to nucleoproteins by electrostatic forces This diffusion was regarded as complete when the quotient of the fluorescence intensities at 590 and 530 nm ($F_{590}/F_{530} = \alpha$, which is a function of the AO P ratio see Chapter 3) for DNP had become constant As Fig 16 shows the biggest decrease for DNP was found within the first 15 minutes at 20 and an asymptotic value was reached after 30 minutes Since however the

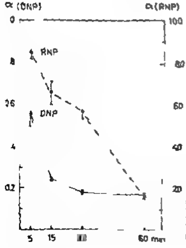


Fig 16 Relation between the diffusion time and α of DNP AO (———) and RNP AO (— — —) complexes Citric acid Na_2HPO_4 buffer pH = 4 1 μ = 0 24

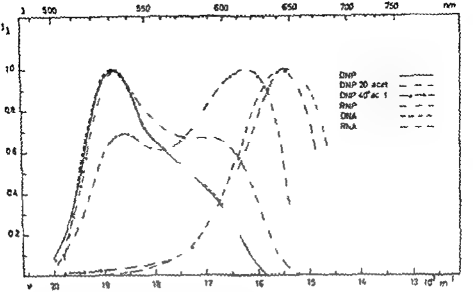


Fig 17 Emission spectra of DNP AO complexes before (DNP) and after blocking of the amino groups of the protein component by acetic acid anhydride at 20 and 40 C (DNP 20 acet DNP 40 acet) Emission spectra of RNP AO DNA AO and RNA AO complexes Citric acid Na_2HPO_4 buffer pH = 6 5 μ = 0 24

RNP droplets started to show elution of the ribosomal particles after 30 minutes the diffusion process was confined to 15 minutes

These staining conditions which were essentially the same as in the investigation of the pure nucleic acids are described in more detail in Chapter 1 The following results were obtained When DNP and RNP were stained at pH 6 5 the emission spectra of the DNP AO and RNP AO complexes proved to be very similar to those of the DNA AO and RNA AO complexes The DNP AO complex like the DNA AO complex showed an emission maximum at 18 800 cm^{-1} and a broad shoulder around 17 200—17 000 cm^{-1} For the RNP AO complex the peak at 18 800 cm^{-1} was entirely absent the emission maximum being at 15 500 cm^{-1} which is comparable to the RNA AO complex (Fig 17) On the other hand distinct differences were found between the RNP AO complex and the RNA AO complex at pH 4 1 Whereas the picture for the DNP AO complex was the same at pH 4 1 as at 6 5 the emission maximum of the RNP AO complex shifted to 16 100 cm^{-1} and, unlike at pH 6 5 had a very prominent side peak around 18 400 cm^{-1} (Fig 18) This side peak which is caused by an increase of the emission band at 18 800 cm^{-1} characteristic for the monomer state of AO indicates that AO must have bound in a monomer molecular form as well which was not the case at pH 6 5 In keeping with

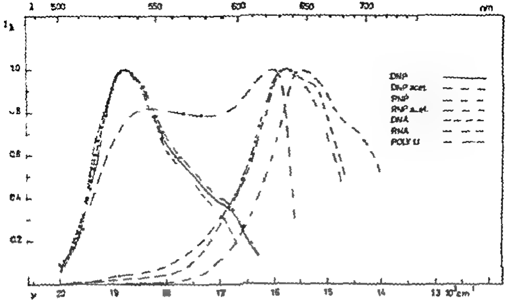


Fig 18 Emission spectra of DNP AO and RNP AO complexes before (DNP RNP) and after blocking the basic amino groups of the protein component by acetic acid anhydride at 20 C (DNP acet, RNP acet) Emission spectra of DNA AO RNA AO and Poly U AO complexes pH = 4 1 μ = 0 6

the argument earlier in this chapter this is best explained by an increased binding of the protein fraction of RNP to the RNA as a result, the dye molecules are prohibited to form all the way associates since neighbouring binding sites of the nucleic acid are largely blocked by the interaction with the protein In the case of DNP a stronger binding of the protein would not change the intensity distribution of the emission spectrum, since free DNA binds AO already in its monomer from a reduced binding of AO in its monomer form would uniformly decrease all emission bands and not affect their intensity distribution

To test this assumption the amino groups of the concomitant proteins were blocked by acetic acid anhydride The side peak of the emission spectrum of the RNP AO complex disappeared (see Fig 18) and the emission maximum shifted to 15 700 cm^{-1} which is where the emission maximum of the corresponding RNA AO complex is to be found On the whole only minor differences were seen between the emission spectra of the RNP AO and RNA AO complexes indicating that probably all binding sites of the nucleic acid chain became available for AO As assumed there was no significant difference between the emission spectra of DNP before and after acetylation both spectra agree well with the emission spectrum of the DNA AO complex, which was recorded as a reference

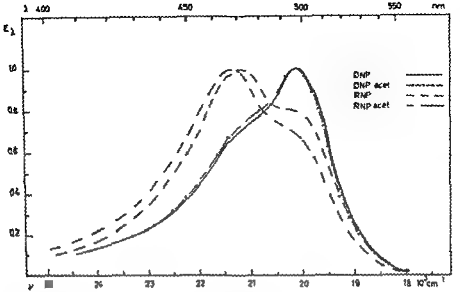


Fig 19 Absorption spectra of DNP AO and RNP AO complexes before and after blocking the amino groups of the protein component by acetic acid anhydride at 20 C pH = 4 1 μ = 0 6

The absorption spectra of the nucleoprotein AO complexes obtained at pH 4 1 like those of the nucleic acid AO complexes are the reverse image of the emission spectra (Fig 19) Irrespective of whether or not the histone amino groups were blocked the DNP AO complex showed an absorption maximum at 20 050 cm⁻¹ and a shoulder around 21 300 cm⁻¹ The RNP AO complex showed a maximum at 21 200 cm⁻¹ and a shoulder around 20 000 cm⁻¹ before acetylation while after acetylation the maximum shifted to 21 400 cm⁻¹ and the shoulder at 20 000 cm⁻¹ decreased

A plot of the centers of gravity for the emission and absorption bands which comprise the different emission and absorption peaks and shoulders (Fig 20)

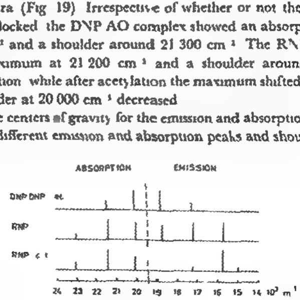


Fig 20 Schematic representation of the position and the relative intensity of the absorption and emission bands of DNP AO and RNP AO complexes before and after blocking the amino groups of the protein component by acetic acid anhydride

revealed the same symmetry for the DNP AO complex as for the DNA AO complex, the emission band at 18,800 cm⁻¹ and the absorption band at 20,000 cm⁻¹ are maximal, which is characteristic for the monomer molecular form of AO. For the native RNP AO complex the symmetry is also quite good, although the band at 15,700 cm⁻¹ has no real equivalent on the absorption side. Since the emission and absorption bands at 18 000 cm⁻¹ and 20,000 cm⁻¹ respectively are strikingly high, a dye binding in the monomer form has to be assumed here as well; on the other hand, the band at 15 700 cm⁻¹ points to the existence of associated dye molecules. After blockage of the amino groups of the ribosomal protein there is no real symmetry between emission and absorption. The monomer emission band gives way to the emission band characteristic for associated dye molecules. As in the case of the nucleic acid AO complexes, as long as the symmetry in absorption and emission is maintained, the different bands express the transition from the ground state to an excited electronic state and *vice versa*, obeying the Franck Condon principle. The change in the band intensities seen in the RNP AO complex indicates an increased transition probability in the region of the long wave emission and the short wave absorption and is a result of an increasing approach of the dye molecules. If however the dye molecules form higher associates, the emission bands in the long wave region lose their partners on the absorption side. This can be explained by a non radiative loss of energy leading to an intermediate state between the excited and the ground state; the emission then takes place during the transition from this intermediate metastable state to the ground state; since the energy difference between the metastable and the ground state is smaller than that between the excited and the ground state, the emission shows a lower frequency than that caused by a transition from the excited to the ground state.

Summarizing the spectral data, the spectral distributions of the emission and absorption of nucleoprotein complexes depend not only on the structure of the nucleic acid but also on the binding strength of the protein fraction to this acid. When the interaction between protein and nucleic acid is minimized by using neutral pH or by blocking the amino groups of the protein, the spectral distribution is comparable to that of pure nucleic acid AO complexes. The DNA in the DNP complex can be assumed to have the same degree of structural organization as the isolated DNA, *i.e.* that of a double stranded helix. Also the organization of the RNA structure in the RNA complex must be about the same as in the isolated RNA. As in the case of the isolated RNA it cannot be regarded as being as low as in Poly U, which shows additional emission bands of its AO complex between 15,000 cm⁻¹ and 14 500 cm⁻¹ (Fig. 8 and Fig. 18) indicating a higher dye association and consequently a lower structural organization than found in RNA of the RNP complex. If however the protein

fraction in the nucleoprotein interferes with the binding of AO to the nucleic acid as is the case at acid pH the emission spectrum and the absorption spectrum will cease to give a true picture of the organization of the nucleic acid structure This is especially the case for ribonucleoproteins

B Contribution of histones and ribosomal proteins to the fluorescence of DNP-AO and RNP AO complexes

Since proteins as already mentioned can also show anionic charges that might bind the cationic AO it was necessary to investigate the interaction between isolated histones and ribosomal proteins before trying to evaluate a quantitative study of the AO binding to nucleoproteins Moreover proteins show not only a fluorescence in the long wave ultraviolet (between 300 and 350 nm Teale 1960) but also a luminescence in the violet and in the visible region above 500 nm when excited above 310 nm (Dumartin *et al* 1957) this type of luminescence has been explained as a phosphorescence induced by forbidden absorption bands A similar luminescence was observed when cell proteins (from mouse fibroblast cells) were excited at 365 nm The emission spectrum of these cell proteins overlaps that of AO consequently it was also desirable to know the luminescence intensity of histones and ribosomal proteins (which constitute a major part of the cell proteins) in this overlapping region as this might contribute to the fluorescence of the nucleoprotein AO complexes

In order to isolate the histones and ribosomal proteins the nucleic acids were extracted from DNP and RNP microdroplets sprayed onto glass slides using 5 per cent TCA at 90 C for 15 minutes The fluorescence intensities at 530 and 590 nm were estimated before and after AO staining at different pH s the results were correlated to the dry mass of the protein droplets To facilitate comparisons with the fluorescence intensities obtained from the nucleoproteins after AO staining the fluorescence intensities of the unstained and stained histone and ribosomal microdroplets have been expressed in per cent of the fluorescence intensities of the respective nucleoprotein stained with AO under the same conditions

As Table III shows the fluorescence of histones before and after AO staining never exceeded about 4 per cent of the DNP AO complex s fluorescence intensity at 530 nm (18 870 cm $^{-1}$) or about 6 per cent at 590 nm (16 950 cm $^{-1}$) The fluorescence intensity of the ribosomal proteins on the other hand was about 30 per cent stained and unstained at 530 nm — but not more than about 5 per cent at 590 nm — of the fluorescence intensity of the RNP AO complex at pH 4 1 At pH 6 5 the percentages for unstained ribosomal protein

were somewhat less (25 and 4 per cent). After AO staining, however, the fluorescence intensities doubled for both wave lengths.

This high contribution from ribosomal protein to the RNP AO fluorescence at 530 nm is explained by the fact that the total fluorescence intensity of the RNP AO complex at 530 nm is very low compared to the DNP AO complex; based on the dry weight, the fluorescence intensity of the unstained ribosomal protein was the same as that of the corresponding histone at both wave lengths and pH's investigated. The data in Table III also indicate that ribosomal protein does not bind significant amounts of AO at pH 4 since the fluorescence intensities before and after staining with AO were about the same (the standard error of these determinations varied between 2 and 7 per cent of the mean). On the other hand, the high increase of the fluorescence intensity after AO staining at pH 6.5 indicates that a significant amount of AO must have been bound to the acidic groups of the ribosomal protein; the binding of AO to ribosomal protein accounted for at least 25 to 30 per cent of the fluorescence intensity at 530 nm. This finding contradicts the assumption of Morgan and

TABLE III. Fluorescence intensity at 530 nm and 590 nm of the protein part of DNP and RNP before and after staining with acridine orange in per cent of the fluorescence intensity of the total DNP AO and RNP AO complex. Fluorescence intensity of the acridine orange stained gelatine part in per cent of the fluorescence intensity of the total DNA gelatine-AO and RNA-gelatine AO complex.

	pH 4.1				pH 6.5			
	Protein fluorescence intensity in per cent of the total complex at		Protein AO fluorescence intensity in per cent of the total complex at		Protein fluorescence intensity in per cent of the total complex at		Protein AO fluorescence intensity in per cent of the total complex at	
Total complex	530 nm	590 nm	530 nm	590 nm	530 nm	590 nm	530 nm	590 nm
DNA-Gelatine AO			0.1	0.2			0.1	0.2
DNP AO	2.2	3.1	3.9	6.2	0.9	1.5	1.8	4.5
DNP 20 acet AO	1.4	1.6	2.3	2.9	1.2	1.6	2.3	4.6
RNA-Gelatine AO			2.7	0.2			3.1	0.1
RNP AO	30.6	5.3	28.6	4.3	24.6	2.8	59.0	6.7
RNP 20 acet AO	35.7	3.6	35.7	3.6	23.4	3.6	64.1	10.0

Rhoads (1965) that a binding of AO to ribosomal proteins can be disregarded even at a neutral pH

By way of comparison, the fluorescence intensity of the AO stained gelatine used in the nucleic acid microdroplets (see Chapter 3) contributed at pH 6 5 only about 3 per cent of the fluorescence intensity of the RNA Gelatine AO complex at 530 nm The maximal value obtained was 7 per cent (for the Poly U Gelatine AO complex at 530 nm)

The ratios between the fluorescence intensities of gelatine histone and ribosomal protein per dry weight at 530 nm after staining with AO at pH 6 5 work out as 1 18 7 22 9 Surprisingly the relative amounts of acidic amino acids which are supposed to bind the AO do not show such correspondingly large variations Thus the figures reported are for gelatine 11 2 mole per cent (Tristram 1949) for histone 13 8 mole per cent (Crampton *et al* 1957) and for ribosomal protein 18 8 mole per cent (Cohn and Simson 1963) The biggest difference between gelatine and the two other proteins lies however in the content of aromatic amino acids which is about five times lower in gelatine than in the other two proteins The connection between the low fluorescence intensity after AO staining and the low content of aromatic amino acids may be partly due to the fact that the emission from proteins in the blue region (*i e* from the aromatic amino acids) overlaps the region of AO absorption Under such conditions an energy transfer is possible from aromatic amino acids to the bound AO this might increase the fluorescence intensity (fluorescence efficiency) of AO bound to histone and ribosomal protein in comparison to that of AO bound to gelatine It is conceivable therefore that the fluorescence intensity of AO stained histones and proteins is not related solely to the amount of AO bound but may be influenced by other processes as well for instance energy transfer from proteins to the dye

Summing up at acid as well as at neutral pH s histones contribute only a negligible percentage of the total fluorescence of the DNP AO complex Ribosomal proteins however contribute 30 per cent of the RNP AO fluorescence intensity at 530 nm and about 5 per cent at 590 nm at pH 4 1 this contribution seems to be entirely due to the proteins autoluminescence At pH 6 5 however a significant amount of AO is bound which increases the contribution of the ribosomal protein fluorescence to 60 per cent at 530 nm The increased dye uptake of the ribosomal proteins is explainable by an increased dissociation of the acid (carboxyl) groups at neutral pH

C Quantitative aspects of the binding of acridine orange to nucleoproteins

1 *Elimination of the protein influence*

The quantitative AO binding of nucleoproteins has been investigated primarily at pH 4. At this pH only the binding to nucleic acids need be considered, the binding to histones or ribosomal proteins being negligible. As will be shown, the same is true when intracellular nucleoproteins are stained with AO. The ionic strength used was that which proved suitable for the binding of AO to whole cells. For this reason, all the results discussed in this section are directly applicable to AO stained cells or cell particles.

In order to make the results of AO binding to nucleoproteins comparable with those when AO binds to nucleic acids, the fluorescence of the DNP AO and RNP AO complexes measured at 530 and 590 nm were corrected for the fluorescence intensities contributed by the histones and the ribosomal proteins.

The fluorescence intensity at 530 nm (F_{530}) of the DNP AO and the RNP AO complexes under different experimental conditions was again related to the nucleic acid phosphorus content ($F_{530}/10^{-12}$ mole P) and plotted against the quotient $F_{590}/F_{530} = \alpha$ as was done for the nucleic acid AO complexes (Fig. 21). The nucleoproteins were studied in their native state and after blocking the amino groups of the concomitant proteins with acetic acid at 20° and 40 °C for 15 minutes. When the regression line and the 95 per cent confidence limits are determined for the values of the nucleoprotein AO complexes treated with acetic acid anhydride, the following observations can be made: (a) the native DNP AO and RNP AO complexes are found beyond the confidence limits of the regression line; (b) the function (Fig. 21) which can be written as

$$\alpha = 22.3\,(F_{530}/10^{-12}\ \text{mole P})^{-0.64} \qquad (20)$$

has a shape similar to that characteristic for the nucleic acid AO complexes at the same pH and ionic strength (pH 4.1, μ 0.6) (see equation (16), Chapter 3). In fact there were no significant differences between the two relations: the function for nucleoprotein AO complexes falls almost entirely within the confidence limits of the corresponding function for nucleic acid AO complexes.

Compared with the acetylated nucleoprotein AO complexes, the native DNP AO complexes show lower fluorescence intensities at 530 nm than one would expect from their α values, assuming that function (20) is valid in this case as well. This behaviour indicates that not all phosphate groups capable of binding AO have become available for the dye, as in the case of the acetylated nucleoprotein AO complexes. No significant differences were found between the fluorescence intensities of the nucleoprotein AO complexes after treatment

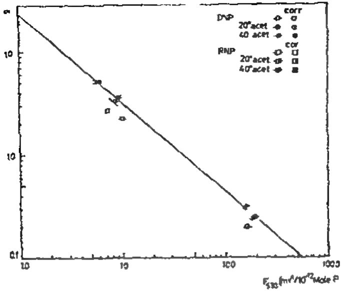


Fig 21 Graphs showing α (ratio of the fluorescence intensities at 590 and 530 nm (F_{590}/F_{530}) for the binding of acridine orange to DNP and RNP before (○ ○ □ -□-) and after blocking the amino groups of the protein component by acetic acid anhydride at 20 (◑ ◑ ◧ -◧) and 40 C (● ● ■ ■) as a function of the fluorescence intensity at 530 nm per 10 ¹ mole nucleic acid phosphorus (F , [mV]/10 mole P) pH = 4 1 μ = 0 6 Regression line and 95 per cent confidence intervals of the function Values with (○ ◑ ● □◧■) and without (-○ -◑ ● -□ ◧ ■) correction for the protein AO fluorescence (luminescence of the protein plus fluorescence of protein bound acridine orange)

with acetic acid anhydride and the nucleic acid AO complexes (see also Table IV) This indicates that the amount of phosphate groups in nucleo proteins available for the dye is the same as in isolated nucleic acids (i e all phosphate groups of the nucleoprotein complexes) Moreover, there seems to be the same functional dependence between α and the degree of structural order as is found in nucleic acid AO complexes

When the temperature of the blocking reaction with acetic acid anhydrid at 20 C was increased by 20 so that the velocity of the reaction increased by a factor of 4 to 8 (van t Hoff s law) the values for α and $F_{530}/10^{12}$ mole P still fit function (20) This indicates that although the blocking of amino groups was accelerated no additional phosphate groups which could interact with AO were in fact liberated had this been the case the respective values would be shifted to the right side of function (20) in the diagram in Fig 21 Apparently the acetylation at 20 has already blocked all protein amino groups For the DNP AO complex however a significant rise in α accompanied the acetylation procedure and even more so an increase of the reaction temperature (Table IV)

the amount of helix regions at neutral pH was estimated to be not more than 5 per cent Poly U, which was taken as a random coil reference for nucleic acid AO complexes stained at pH ▮ 5, does not seem to be suitable as a standard since, like Poly A, it gives a bad fit to equation (16) in Chapter 3 and equation (20) in Chapter 4 Although the differences in the emission spectra between RNP AO and Poly U AO complexes (Fig 18) suggests that the RNA in the RNP complex is not random coiled to the same extent as Poly U, only a small error may be involved in regarding the RNA in the acetylated RNP as completely single stranded Accordingly, the α for RNP acetylated at 20° C was used as the standard for a single stranded (random) coil structure (α_S = 4 89) The ratio S/D between single stranded coil regions and double stranded helix regions is in analogy with the isolated nucleic acids given by the equation

$$S\,D = \frac{\alpha - \alpha_D}{\alpha_S - \alpha}\quad C$$

where $\alpha \;= I_{640}/F_{530}$ for the nucleoprotein in question,

$\alpha_D = f^D_{640}/f^D_{530}$ for the acetylated double stranded helix DNP AO complex,

$\alpha_S = f^S_{640}/f^S_{530}$ for the acetylated single stranded coil RNP AO complex

The fluorescence coefficients $f_{530} = I_{530}/10^{-12}$ mole P required for calculating C can be determined for any value of α from equation (20), which may be reversed

$$F_{530}/10^{-12}\text{ mole P} - \left(\frac{22\ 3}{\alpha}\right)^{1\ 16} \qquad (20a)$$

C which is defined as the ratio of the fluorescence coefficients for double stranded to single stranded nucleic acids at 530 nm f^D_{530}/f^S_{530} can be directly computed from α_D and α_S since

$$C = \frac{F^D_{530}/10^{-12}\text{ mole P}}{I^S_{530}/10^{-12}\text{ mole P}} = \left(\frac{\alpha_S}{\alpha_D}\right)^{1\ 16} \qquad (21)$$

If α_D = 0 140 and α_S = 4 89 then C = 61 9

These values give the following relative amounts of double stranded DNA like helix regions and single stranded RNA like coil regions in DNP after acetylation (which increases α)

	α	Helix regions	Coil regions %
DNP 20 C acet	0 212	43	57
DNP 40 C acet	▮ 301	31	69

This result also means that under the same conditions twice the amount of helix regions are destroyed in isolated DNP as in intracellular DNP, which served as the standard in the calculation. Consequently the helix structure of DNP must be far less stable in the isolated than in the intracellular state. There is another difference. The fluorescence intensity at 530 nm of the isolated native DNP complex increases approximately by a factor of 1.5 after acetylation at 20° C when compared with the fluorescence intensity at 530 nm after acetylation which was extrapolated for the same α value as in the native DNP complex (0.196) according to equation (20) (see Fig. 21); the corresponding increase for intracellular DNP, however, is by a factor of 4 (see Chapter 6). This means that the part of the AO binding anionic phosphate groups that is blocked is much bigger in intracellular than in isolated DNP, which is already almost completely liberated. This is understandable if one assumes that during the isolation procedure a certain amount of proteins is lost which neutralizes the phosphate groups of DNP within the cell (Peacocke, 1960).

3. *Determination of the ratio, the relative and the absolute amounts of DNP and RNP in mixtures*

Equations (17), (18) and (19) in Chapter 3 also permit the calculation of the ratio, the relative and the absolute amounts of two nucleic acids or two nucleoproteins in mixtures, provided that they differ in the degree of structural order, *e.g.* DNA and RNA or DNP and RNP.

By analogy with the nucleic acids, the fluorescence intensities for *e.g.* DNP and RNP acetylated at 20° C at 530 nm (F_{530}, given in mV amplified photomultiplier signal) and 590 nm (F_{590}) are given by

$$F_{530} = \mathrm{DNP}\, f_{530}^{\mathrm{DNP}} + \mathrm{RNP}\, f_{530}^{\mathrm{RNP}} \quad (22)$$

$$F_{590} = \mathrm{DNP}\, f_{590}^{\mathrm{DNP}} + \mathrm{RNP}\, f_{590}^{\mathrm{RNP}} \quad (23)$$

where DNP, RNP = the amount of DNP and RNP given in 10^{-12} mole P
$f_{530,590}^{\mathrm{DNP}}$, $f_{530,590}^{\mathrm{RNP}}$ = fluorescence coefficients for DNP and RNP at 530 and 590 nm given in mV/10^{-12} mole P

As already mentioned, DNP and RNP bind AO stoichiometrically. The fluorescence coefficients can be read off the diagram in Fig. 22 in this special case, for which the fluorescence coefficients have been determined directly. If, however, only α_{DNP} and α_{RNP} are known, the coefficients may be determined as before from equation (20a).

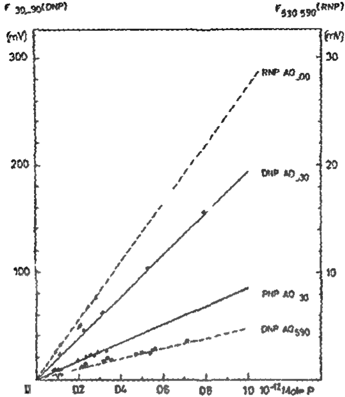


Fig. [illegible] Graph showing the stoichiometric relation between the fluorescence intensity at wavelength 530 nm and 590 nm (F_{530}, F_{590}) and the phosphorus content of DNP AO and RNP AO complexes. Protein amino groups blocked by acetic acid anhydride at 20 °C, pH 4.1, $\mu = 0.6$

a) the ratio is given by

$$\mathrm{RNP/DNP} = \frac{a - a_{DNP}}{a_{RNP} - a}\ C \quad (24)$$

where $a = F_{530}/F_{590}$ for any RNP/DNP ratio

$a_{DNP} = f_{530}^{DNP}/f_{590}^{DNP}$ for pure DNP,

$a_{RNP} = f_{530}^{RNP}/f_{590}^{RNP}$ for pure RNP,

$C = f_{530}^{DNP}/f_{530}^{RNP}$

C can be determined from the fluorescence coefficients or in analogy with equation (21)

$$C = \left(\frac{x_{RNP}}{x_{DNP}}\right)^{[illegible]} \quad (25)$$

(b) the relative amounts of DNP and RNP, in millivolts (mV) amplified photomultiplier signal, are given by

$$\mathrm{DNP} = \frac{F_{530}}{1 + a}\ [\mathrm{mV}],\quad a = \frac{\alpha - \alpha_{\mathrm{DNP}}}{\alpha_{\mathrm{RNP}} - \alpha} \quad (26)$$

$$\mathrm{RNP} = \mathrm{DNP}\ a\ C \quad [\mathrm{mV}] \quad (27)$$

(c) the absolute amounts of DNP and RNP in 10^{-11} mole P are obtained by dividing the relative values by the fluorescence coefficient f_{530}^{DNP}

$$\mathrm{DNP}\ [10^{-11}\ \mathrm{mole\ P}] = \mathrm{DNP}\ [\mathrm{mV}]/f_{530}^{\mathrm{DNP}}\ [\mathrm{mV}/10^{-11}\ \mathrm{mole\ P}] \quad (28)$$

$$\mathrm{RNP}\ [10^{-11}\ \mathrm{mole\ P}] = \mathrm{DNP}\ [10^{-11}\ \mathrm{mole\ P}]\ a\ C \quad (29)$$

This calculation refers primarily to the case in which all phosphate groups of the DNP and RNP complexes are available for AO *i e* for acetylated DNP and RNP However it is also applicable when only part of the AO binding phosphate groups are available as was found to be the case with DNP This is in theory possible because the blocking of DNA phosphate groups by protein amino groups does not alter the mode of dye binding (monomer molecular form) and hence the emission spectrum The amount of free unblocked AO binding phosphate groups (DNA PO_4) may be calculated by using the fluorescence coefficient for DNP with completely free phosphate groups this can be computed from equation (20a) using the α for DNA with blocked phosphate groups Such a procedure would not be applicable in the case of a blocking of AO binding RNP phosphate groups Phosphate blocking alters the mode of dye binding (associated molecular form into monomer molecular form) and hence the emission spectrum The fluorescence coefficient is no longer comparable to an α which has been altered not by changes in the structural organization but by an exclusion of the phosphate groups from the dye binding

Equation (19) was tested for a mixture of RNA and DNA by comparing the actual ratio RNA/DNA (w/w) with the expression $\frac{\alpha - \alpha_{\mathrm{DNA}}}{\alpha_{\mathrm{RNA}} - \alpha}$ were α is the quotient F_{640}/F_{530} for any RNA/DNA ratio and α_{DNA} and α_{RNA} the corresponding values for pure DNA and RNA As expected a linear relationship was found (see Fig 23) This plot was used originally to determine the size of C in an earlier investigation at lower ionic strength (Rigler Jr 1963 1964) C is given as the intercept between the regression line and the abscissa when $\frac{\alpha - \alpha_{\mathrm{DNA}}}{\alpha_{\mathrm{RNA}} - \alpha}$ equals unity

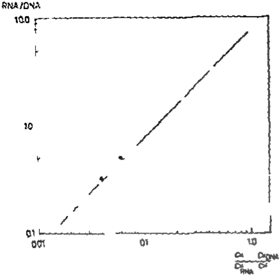


Fig 23 Relationship of the RNA/DNA (w/w) ratio of different DNA RNA mixtures to the expression $\frac{\alpha - \alpha_{DNA}}{\alpha_{RNA} - \alpha}$ pH = 4 1 μ = 0 006

If for instance, the ratio and the amounts of DNA and RNP acetylated at 20° C in a mixture are to be determined the values 0 242 and 4 89 (see Table IV) must be used for a_{DNP} and a_{RNP} C is then calculated as 32 8

The absolute amounts of DNP and RNP (in 10^{-12} mole P or DNA PO_4 and RNA PO_4) are determined with the fluorescence coefficient for DNP f^{DNP}_{530} = 190 5 mV/10 ^{12} mole P

When it comes to determining the ratio of ordered to disordered structures or the amount of DNA PO_4 and RNA PO_4 in a mixture of DNP and RNP within cells or cell structures it would be tedious and complicated to correct for the protein fluorescence of the DNP AO and RNP AO complexes Consequently a test was made of the error involved in using the uncorrected values It was found that after acetylation at 20 and 40 C, these values fit equation 20 as well as do the corrected values (Fig 21) although the a values are diminished as in the case of the RNP AO complex (Table V)

If the relative amounts of helix and coil regions for say the DNA or DNP within a cell are to be determined the values a_S = 0 140 a_D = 3 31 and C = 59 1 are taken The amount of helix regions of isolated DNP acetylated at 20° and 40 C determined from uncorrected and corrected values, respectively differed by less than 5 per cent On the other hand to determine the amount of DNA PO_4 and RNA PO_4 in a mixture of DNP and RNP — in which, say the RNA is completely single stranded and the DNA has about 60 per cent single strands (as in the case of the isolated and acetylated RNP and DNP fractions — the relevant values would be a_{DNP} = 0 244 a_{RNP} = 3 31 and C = 20 6

TABLE V Fluorescence coefficients at 530 nm (f_s) and a for acetylated DNP AO and RNP AO complexes. No correction for protein AO fluorescence. pH 4.1 $\mu = 0.6$

	f_s (mV/10 ¹¹ mole P) Mean ± S.E.	a Mean ± S.E.
DNP intracellular 20 °C acet	359.0	0.140 ± 0.003
DNP isolated 20 °C acet	191.8 ± 3.9	0.244 ± 0.003
DNP isolated 40 °C acet	158.9 ± 6.0	0.308 ± 0.003
RNP isolated 20 °C acet	8.61 ± 0.31	3.31 ± 0.12
RNP isolated 40 °C acet	9.07 ± 0.39	3.51 ± 0.31

Summary

The binding of AO to nucleic acids in nucleoproteins is influenced by the amino groups of the concomitant protein, indicating competition between the dye and the amino groups for binding sites on the nucleic acids. When this interference by amino groups is excluded, either by decreasing their positive charge through a heightening of the pH or by blocking them with acetic acid anhydride, the emission and absorption spectra of DNP AO and RNP AO complexes agree with those of the DNA AO and RNA AO complexes. Thus the DNP AO complex shows prevailing bands at 20 030 cm⁻¹ in the absorption and at 18 800 cm⁻¹ in the emission spectrum, indicating a dye binding in a monomer molecular form characteristic for helix structures. The RNP AO complex exhibits predominant bands at 21 400 cm⁻¹ in the absorption and at 15 700 cm⁻¹ in the emission spectrum, suggesting dye binding in the associated molecular form characteristic for random coil structures. Although the degree of chain organization shown by RNA in RNP is very low, it is not as low as in Poly U; this is indicated by distinct differences between the emission spectra of the two nucleic acids AO complexes.

The greater interaction between proteins and nucleic acids at an acid pH is indicated by a change in the emission spectrum of the RNP AO complex. The emission band at 18 800 cm⁻¹ is very accentuated, which is not the case when the protein nucleic acid interaction is minimal. This points to a dye binding in the monomer molecular form at the expense of the associated. No such change is displayed by the DNP AO spectra on increasing protein nucleic acid interaction, since AO binds to isolated DNA already in the monomer form.

Quantitative studies have been performed at an acid pH (4.1) in order to avoid binding of AO to the anionic groups of the histones and ribosomal

proteins When the amino groups of the proteins are blocked with acetic acid anhydride, DNP and RNP behave like the free nucleic acids in binding AO Changes in the emission spectrum can then be ascribed to differences in the structural order of the nucleic acid chains Consequently the amount of helix and coil regions in isolated nucleoproteins can be determined as in free nucleic acids, using the quotient α (F_{580}/F_{530}) of the AO complexes In the case of a mixture of two nucleoproteins or of nucleic acids of differing chain order, α can be used to determine the ratio as well as the relative and absolute amounts of both nucleoproteins or nucleic acids The equations for these calculations are derived and examples given

CHAPTER 5

The Kinetics of Acridine Orange Binding to Intracellular Nucleoproteins

A General considerations

When using a binding reaction mediated by electrostatic forces one needs exact knowledge of the kinetics and the conditions of this reaction, if reproducible results are to be obtained The staining of cell components by cationic or anionic dyes may be regarded as a diffusion process, in which the dye ions diffuse from the concentrated staining solution through the cell membrane into the cell

Assuming that the cell membrane is impermeable for the anionic macromolecules within the cell and permeable for the cationic dye molecules, the number of dye cations will exceed the number of the macromolecules anionic charges after diffusion equilibrium has been attained, even if the original dye concentration was lower than the concentration of the macromolecules inside the cell membrane according to the principle of electroneutrality, the charge of dye cations within the cell membrane must equal the negative charge of the macromolecules and other diffusible anions which accompany the dye cations In the case of high macromolecule and low dye concentrations this will lead to a Donnan equilibrium with a higher dye concentration inside the cell than outside

Since dye ions in excess of the macromolecules charge and hence not bound by electrostatic forces would not reflect the true properties of the macromolecules *i e* nucleic acids as discussed in earlier chapters, they have to be separated from the macromolecules by an additional diffusion process The two diffusion processes — usually referred to as staining and differentiation — both obey Fick's diffusion law *i.e* the number of molecules dn diffusing from one compartment through the cross section q to another is proportional to the concentration gradient $\frac{dc}{dx}$ the time dt and the diffusion constant D so that

$$dn = -Dq\frac{dc}{dx}dt$$

To be reproducible a staining process for quantitative analysis must obey the parameters of Fick's equation

proteins When the amino groups of the proteins are blocked with acetic acid anhydride, DNP and RNP behave like the free nucleic acids in binding AO Changes in the emission spectrum can then be ascribed to differences in the structural order of the nucleic acid chains Consequently the amount of helix and coil regions in isolated nucleoproteins can be determined as in free nucleic acids, using the quotient α (F_{530}/F_{640}) of the AO complexes In the case of a mixture of two nucleoproteins or of nucleic acids of differing chain order, α can be used to determine the ratio as well as the relative and absolute amounts of both nucleoproteins or nucleic acids The equations for these calculations are derived and examples given

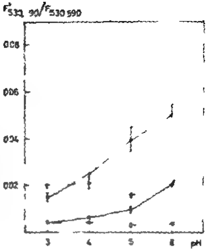


Fig 24 Binding of AO to the protein component of a mouse fibroblast cell (cell line L-929) Average fluorescence intensity at 530 and 590 nm of the protein rest after enzymatic extraction of the nucleic acids (F mean ± standard error of 30 measuring fields 4 to 5 cells each) before (● ● 530 nm ○ ○ 590 nm) and after (—●—●— 530 nm —○—○— 590 nm) AO staining expressed as the fraction of the average fluorescence intensities of an AO-stained mouse fibroblast cell containing nucleic acids (F)

protein droplets as otherwise they would have been dissolved from the glass slides) The fluorescence was again recorded before and after staining the protein residual of mouse fibroplast cells with AO at different pH As shown in Fig 24 no distinct difference was found between the fluorescence before and after AO staining up to pH 4 whereas the protein fluorescence was significantly greater after than before AO staining at higher pH The protein fluorescence at 530 nm and pH 4 amounted to about 2 per cent of the total fluorescence intensity of the AO stained cells containing nucleic acids and consequently may be ignored at 590 nm this contribution was even lower

For this reason all subsequent investigations were carried out at pH 4 1 An increase in the ionic strength of the buffer applied caused a rapid increase of the fluorescence intensity at 530 nm and a simultaneous decrease in a (see Fig 25) Since a is a measure of the amount of dye bound per nucleotid unit (see Chapter 3) and hence of the dye aggregation on the nucleic acid chains this means that the dye binding and aggregation are inversely proportional to the ionic strength The change in a given in Fig 25 relates however to the mixture of DNP and RNP As Fig 13 shows the decrease in a *with increasing ionic strength was relatively greater for the DNP AO complex* than for the RNP AO complex Whereas the quotient between the a s for the RNP AO and DNP AO complexes was about 7 at an ionic strength of 0 006 it increased to about 21 at an ionic strength of 0 6 This means that the spectral differences between the two nucleoprotein AO complexes are much greater at higher ionic strengths a circumstance which is very desirable in the case of quantitative measurements

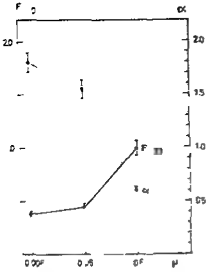


Fig 25 Effect of the ionic strength (μ) of the buffer solution upon the average value of α and the average fluorescence intensity at 530 nm (F_{53}) of a mouse fibroblast cell (mean ± standard error of 50 measuring fields 4 to 5 cells each) Fluorescence intensity at standard staining conditions ($\mu = 0.6$) equals unity pH = 4 1

An increase of the dye concentration led, as one might expect, to an increase of the dye aggregation in the cell, while the fluorescence intensity at 530 nm decreased (Fig 26) However the increase of α also depends upon the time during which the AO is allowed to diffuse back from the cell into the pure buffer solution When ten minutes was given for this rediffusion of AO α doubled during a tenfold increase of the dye concentration However, when the rediffusion time was prolonged to 15 minutes, an increase in dye concentration produced no significant change in α

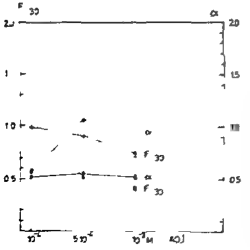


Fig 26 Effect of the AO ion concentration of the staining bath upon the average value of α and the average fluorescence intensity at 530 nm (F_{s}) of a mouse fibroblast cell at 10 minutes (— — —) and 15 minutes (———) rediffusion time Fluorescence intensity at standard staining conditions (10^{-4} M AO 15 minutes rediffusion time) equals unity pH = 4 1 $\mu = 0.6$

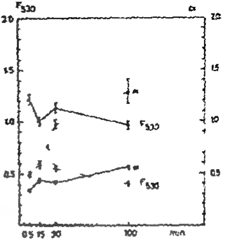


Fig 27 Effect of the staining time upon the average value of α and the average fluorescence intensity at 530 nm (F_{530}) of a mouse fibroblast cell at 10 min (— — —) and 15 min (———) rediffusion time Fluorescence intensity at standard conditions (15 minutes staining time 15 minutes rediffusion time) equals unity pH = 4.1 μ = 0.6

Prolongation of the staining time gave much the same result (Fig 27) Allowing 10 minutes for rediffusion of the dye α increased about 2.6 times when the staining time was prolonged from 5 to 10 minutes When the rediffusion time was increased to 15 minutes however α increased by only about half as much during the same staining time

An increase in the time allowed for the rediffusion of AO from the cell into pure buffer solution following staining led to a rapid decrease in α (Fig 28) and a simultaneous increase in F_{530} Both parameters attained approximately constant values at about 30 minutes indicating that all cations not bound to nucleic acids by electrostatic forces have been separated

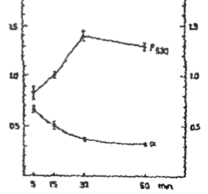


Fig 28 Effect of the rediffusion time upon the average value of α and the average fluorescence intensity at 530 nm (F_{530}) of a mouse fibroblast cell Fluorescence intensity at standard staining conditions (15 min rediffusion time) equals unity pH = 4.1 μ = 0.6

TABLE VI α values of intracellular nucleoprotein AO complexes in mouse fibroblast cells fixed in ethanol (freeze substitution) and in different mixtures of ethanol ether and ethanol acetone

Fixative			α (F_{640}/F_{530}) Mean ± standard error	No of observations
Freeze substitution		24 h	0.35 ± 0.03	30
Ethanol ether	1:1	30 min	0.36 ± 0.10	30
Ethanol acetone	1:3	30 min	0.42 ± 0.01	30
Ethanol acetone	1:1	30 min	0.47 ± 0.01	30
Freeze substitution		96 h	0.50 ± 0.01	30
Ethanol acetone	3:1	30 min	0.56 ± 0.01	30

A practical conclusion from this result is that the use of a sufficiently high ionic strength and allowing at least 15 minutes for the rediffusion of AO from the cell into pure buffer solution renders the degree of dye aggregation (as indicated by the quotient α) nearly independent of the dye concentration and the staining time within certain limits (see Fig 26 and 27) The dye aggregation increases with increasing dye concentration and staining time and decreases with increasing ionic strength and rediffusion time

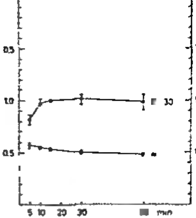
The fluorescence intensity at 530 nm, which is characteristic for the dye in a monomer molecular form changes inversely to α As in nucleic acid and nucleoprotein AO complexes, this indicates an increase in the monomer molecular form at the expense of the associated and *vice versa*

C Influence of the fixation and acetylation

Fixation of the cells is necessary for an undisturbed diffusion of the dye to the intracellular nucleoproteins not affected by an active cellular uptake of the dye However this fixation causes a denaturation of the proteins in the cell membrane and this may influence the membrane's permeability Tests were therefore made with a number of fixation methods involving organic solvents that are known not to cause heavy denaturation of proteins AO staining under constant conditions but using different cell fixatives gave the lowest value for dye aggregation in terms of α for freeze substitution with a rapid temperature exchange (24h) and the highest for fixation in ethanol acetone 3 1 (Table VI)

Low values of α were consistently accompanied by apparent disruption of the cell membrane and a loss of RNP as evidenced by orange red fluorescing particles outside the cell membrane and established objectively by measuring the background fluorescence High values of α were always accompanied by

Fig 29 Effect of the reaction time of acetic acid anhydride at 20 C upon the average value of α and the average fluorescence intensity at 530 nm (F) of a mouse fibroblast cell Fluorescence intensity at standard staining conditions (15 minutes acetic acid anhydride at 20 C) equals unity pH = 4 1 μ = 0 6



marked shrinkage of the cell From this it was concluded that in the former case the high permeability of the cell membrane was due to the damage leading to a rapid diffusion of the dye but also to a loss of nucleoproteins during the staining process in the latter case the low permeability was attributed to shrinkage of the entire cell which would lead to a reduction in the pore size of the cell membrane The apparent damage to the cell membrane from freeze substitution with a rapid temperature exchange may be due to the formation of ice crystals as a result of insufficient substitution of water by ethanol The heavy shrinkage using higher concentrations of ethanol is caused by rapid dehydration of the cells

Two fixation methods caused neither a loss of cell particles as checked by determination of the UV absorption before and after the staining procedure nor visible disruption of the cell membranes nor shrinkage of the cell compared to the native state these were freeze substitution with a slow temperature exchange (from — 70 to room temperature in 96 h) and incubation in a mixture of ethanol acetone 1 1 (v/v) The latter procedure was chosen as being the simpler and also because the visible fine structure after AO staining was even better preserved than it was with freeze substitution

The simultaneous determination of DNP and RNP is most sensitive when these two nucleoproteins AO complexes differ widely in their emission spectra This is the case only if the dye binding phosphate groups are not blocked by the concomitant proteins Accordingly acetylation with acetic acid anhydride in pyridine was adopted as a routine procedure As indicated by the results in Chapter 4 this procedure blocks the amino groups of the proteins so that they cannot interfere with the phosphate groups of the nucleic acid chains involved

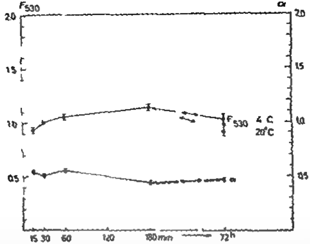


Fig 30 Effect of the fixation time in a mixture of ethanol acetone 1 1 upon the average value of α and the average fluorescence intensity at 530 nm (F_{530}) of a mouse fibroblast cell kept for 180 minutes at 25 C then at − 4 and − 20 C respectively Fluorescence intensity at standard staining conditions (30 minutes fixation time) equals unity pH = 4 1 μ = 0 6

in the binding of AO To minimize the simultaneous splitting of helix structures that may take place if the amino groups of the nucleotide bases are also blocked by acetic acid anhydride the reaction temperature and the reaction time were kept as low and as short as possible (20° C and 15 min) As seen from Fig 29 the increment in F_{530} after 15 minutes reaction time is not augmented by prolonging the reaction time to 60 minutes In other words the blocking reaction at 20 C reaches a limiting value after 15 minutes

A practical problem is the storage of the fixed specimens until staining is performed However no significant change in α and F_{530} was observed when the specimens were kept in the fixation mixture for up to 72 hours at + 4° C (Fig 30) Storing at − 20 C does not seem to give as stable results

D Staining procedure for cells and cell particles

On the basis of these results the following conditions have been applied to the AO staining of intracellular nucleic acids and nucleoproteins in order to achieve a reproducible and quantitative dye binding

Fixation

In ethanol acetone 1 1 for 30 minutes at room temperature after rinsing the cells in isotonic NaCl solution Thereafter immediately staining

Staining at 20 C

1 Blocking of the amino groups that interfere with the nucleic acid phosphate groups, using acetic acid anhydride in pyridine

(a) Pyridine water free	5 min
(b) Acetic acid anhydride pyridine 2 3 (v/v)	15 min

2 Purification from acetic acid anhydride and transfer into an aqueous medium

(a) Ethanol water free	5 min
(b) Ethanol 95 per cent	5 min
(c) Ethanol 60 per cent	5 min
(d) Ethanol 30 per cent	5 min
(e) H_2O redistilled	3 min

3 Staining with 10^{-4} M AO in citric acid Na_2HPO_4 buffer (pH = 4 1 μ = 0 6[1])

(a) Citric acid Na_2HPO_4 buffer	5 min
(b) AO in citric acid Na_2HPO_4 buffer	15 min

4 Rediffusion of the unbound AO

(a) Citric acid Na_2HPO_4 buffer	5 min
(b) Citric acid Na_2HPO_4 buffer	5 min
(c) Citric acid Na_2HPO_4 buffer	5 min

The specimens were sealed with a coverslip and citric acid Na_2HPO_4 buffer of the same pH and ionic strength Besides being related to the pH the emission properties of the bound AO are highly dependent on the size of the solvent s dielectric constant which screens the Coulomb repulsion forces caused by the like charge of the dye molecules (Zanker 1952a) The embedding resins commonly used have far too low a dielectric constant In fact at room temperature only water has a sufficiently high dielectric constant to permit aggregation of the dye molecule which is the basis for the differences in the emission spectra of the various nucleic acid AO complexes . Consequently only aqueous media can be used

Although a complete staining equilibrium was not reached before 30 minutes rediffusion of the unbound dye, this time was confined to 15 minutes mainly because it could not be exceeded in the nucleoprotein AO complexes which were taken as standards for quantitative determinations (see Chapter 4) but also since after 30 minutes rediffusion time elution of RNP from the cells was sometimes observed A possible loss of UV absorbing material during the standard staining procedure was checked by measuring the UV absorption at 265 nm of the same mouse fibroblast cells before and after the staining procedure (no AO which strongly absorbs in the ultraviolet

[1] May be obtained by mixing aliquots of 0 1 M citric acid and 0 2 M Na_2HPO_4

being added to the staining buffer) The average decrease in UV absorption after staining was less than —0 2 per cent of the original value

Summary

The aggregation of AO within an AO stained cell (as indicated by the quotient α and the fluorescence intensity at 530 nm) increases with increasing dye concentration and staining time and decreases with increasing ionic strength and the time during which the unbound AO is allowed to diffuse out of the cell (rediffusion)

The combination of a high ionic strength (0 6) and a rediffusion time of at least 15 minutes renders the dye binding to some extent independent of the dye concentration and the staining time Complete staining equilibrium is attained if rediffusion is permitted for 30 minutes A detailed description of the staining procedure is given

CHAPTER 6

Quantitative Investigations of Intracellular Nucleic Acids and Nucleoproteins

Since the first investigations into the vital staining of plant cells (Bukatsch and Haitinger, 1940 Hofler, 1949) and animal cells (Strugger, 1940 1947 1949) with acridine orange, a vast literature has accumulated about the use of this substance as a stain for vital and fixed biological structures Mention will be made of the studies by Schummelfeder *et al* (1957, 1958), Armstrong (1956 1957) and Bertalanffy (1956 1963) These authors were the first to work out a reproducible staining procedure for animal cells and to prove that the green nuclear fluorescence was due to DNA and the red cytoplasmic to RNA The affinity of acridine orange to nucleic acids had already been indicated by Goessner (1949), Schummelfeder (1950) Zeiger and Harders (1951) De Bruyn *et al* (1953) and Zeiger and Wiede (1954) As a result of the investigations it seemed that the staining technique represented a very powerful tool with which to distinguish between different nucleic acids in biological structures Thus acridine orange was used to study qualitative changes of nucleic acids not only in viruses and bacteriophages (Anderson *et al*, 1959, Mayor 1963) and in tumor cells (Bertalanffy and Bertalanffy, 1960 Bertalanffy, 1962 Sani *et al*, 1963), but also in chromosomes (Nash and Plaut 1964 Wolstenholme 1965) These authors used acridine orange as a stain for fixed cell structures certain aspects of the qualitative and quantitative composition of which are reflected by the stain s binding to nucleic acids Used as a vital stain, acridine orange besides being bound to nucleic acids, is also actively incorporated into the cell This latter aspect has also been used to study a cell s functional performance, *e g* in the pioneer work of Haitinger Strugger and Hofler and in the investigations of Kolbel (1947), Stockinger (1958) Wittekind (1958) Wolf and Aronson (1961) and Robbins and Marcus (1963) concerning the vital staining of animal cells Other interesting views on the vital staining of plant cells have been elaborated by Bancher and Hofler (1959) and Bancher and Holzl (1963)

Most of this work has been based on a more or less subjective examination of color changes and fluorescence intensities and only a few attempts have been made to support and explain these findings on an objective qualitative and quantitative basis *i e* by a careful analysis of the emission spectra and the comparative measurement of the fluorescence intensities on the cell level Inter

esting preliminary work in this field has been done on vitally stained animal cells by Loeser *et al* (1960, 1962) and West (1965), and on vitally stained plant cells by Bancher and Holzl (1963)

It was hoped that a careful study of the physical properties of nucleic acid and nucleoprotein AO complexes would throw more light onto this highly interesting picture of AO stained cells and cell particles and possibly reveal valuable information about the structure and function of nucleic acids Some examples from analyses of the fluorescence emitted by biological structures will be given here on the basis of the results elaborated in the preceeding chapters

A Biological examples

1 Spectral analysis of AO stained cell structures

Before analysing AO stained cell structures containing nucleic acid one had to preclude the occurrence of other substances which might interfere with the AO binding to nucleic acids or nucleoproteins or which themselves behave like nucleic acids To the first group belong positively charged substances like proteins which would in particular affect the intensity distribution of poorly organized nucleic acid AO complexes (see Chapter 4) The reactive protein groups of the RNP particles are easily blocked by the acetylation procedure, unless other proteins are involved which bind more firmly to nucleic acids Unless the influence of the protein component can be excluded one should thus be careful about interpreting say, AO stained virus nucleoprotein complexes Other substances can imitate the properties of either highly or poorly organized nucleic acid structures depending on the frequency and sequence of their dye binding (anionic) groups To the first kind belong proteins with a high number of acidic amino acids *e g* serum albumin or fibrin even at pH 4 these may present anionic groups Then there are phosphatides which occur as lecithine and cephalines in the nervous system as well as in the egg yolk, where the phosphoprotein ovovitellin is also found To the second kind belong poly sulfates such as heparin chondroitin sulfate and mucoitine sulfate Their tight sequence of anionic groups enables them to cause a high aggregation of AO in the same manner as poorly organized nucleic acids In general such undesirable influences can be excluded by the right choice of biological test system, but they must be kept in mind when investigating unknown biological structures

The following figures show the emission spectra of nucleic acid AO complexes in different regions of a mouse fibroblast cell (Fig 31) The nuclear region shows a maximal emission band at 18 800 cm^{-1} characteristic for helix structures,

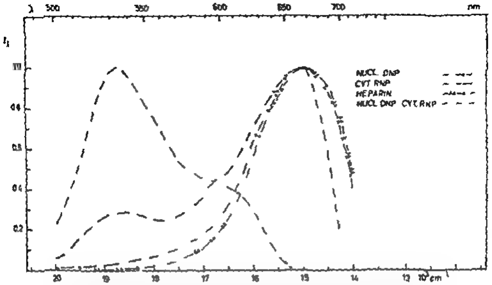


Fig 31 Emission spectra of the nuclear chromatin AO (nucl DNP) and the cytoplasmic AO (cyt RNP) as well as the whole cell AO (nucl DNP — cyt RNP) complexes of a mouse fibroblast cell Emission spectrum of the cytoplasmic AO (Heparin AO) complex of a rat ascites cell Citric acid Na_2HPO_4 buffer pH 4 1 μ = 0 6 15 minutes acetic anhydride at 70 C (standard staining)

while the cytoplasmic region shows a maximal emission band at 15 000 cm^{-1} characteristic for random coil structures While the nuclear emission resembles that of a typical DNA AO complex the cytoplasmic emission is closer to the emission of the Poly L AO complex than to that of RNP AO which shows a maximum at 15 700 cm^{-1} (Fig 18) Since it is very unlikely that the RNP particles situated within the cell have a lower structural organization than the isolated RNP particles in fact this emission spectrum indicates the presence of an additional nucleic acid component with a lower structural organization than the RNA in RNP which probably contributes the distinct shoulder at 15 700 cm^{-1} It is most tempting to assume that this emission spectrum of the cytoplasmic AO complex reflects the structural organization of the polyribosomal complex as found by Warner and Rich (1964) Goodman and Rich (1963) and Gierer (1963) In the electron microscope the ribosomes are seen attached to single thin strands (10—15 Å) messenger RNA at intervals of 50—150 Å The amount of ribosomes per strand ranges from four to five particles up to forty This instance concerns the attachment of a nucleic acid with some degree of structural organization (ribosomal RNA) to another nucleic acid without any structural order (mes

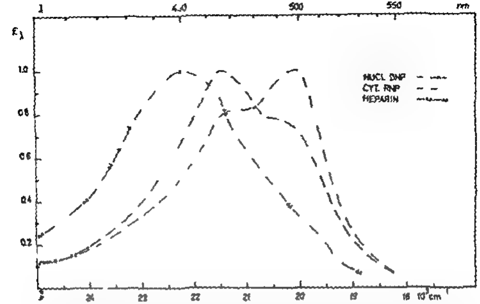


Fig 32 Absorption spectra of the nuclear chromatin AO (nucl DNP) and the cytoplasmic AO (cyt RNP) complexes of a mouse fibroblast cell Absorption spectrum of a cytoplasmic AO (Heparin AO) complex of a rat ascites mast cell Standard staining

senger RNA) The emission spectrum of the total cell comprises the emission of helix and random coil nucleic acid AO complexes This may be compared with the emission spectrum of rat ascites mast cells containing high amounts of heparin (Holmgren and Wilander, 1937) This spectrum is almost identical to that of the cytoplasmic region of the mouse fibroblast cell but here the high aggregation of AO cations is caused, not by a low order nucleic acid chain, but by a polysulphate chain which binds AO in the same way as nucleic acids

The absorption spectra (Fig 32) show the same behaviour in principle as high and low order nucleic acid AO complexes As in the DNA AO and DNP AO complexes the absorption maximum of the nuclear region is at 20,00 cm^{-1}, while that of the cytoplasmic region at 21 500 cm^{-1} agrees well with that of the isolated RNA AO and RNP AO complexes The heparin AO complex of the rat ascites mast cell shows an absorption maximum at 22,200 cm^{-1} which is in good agreement with the findings of Appel and Zanker (1958) for heparin AO complexes in solution

The emission spectra of AO stained T_2 phages indicates the presence of helix structures Fig 33) When the phages are heated to 100 C and rapidly cooled, the emission spectrum of the AO complex is characteristic of a low-order nucleic acid Human metaphase chromosomes prepared by

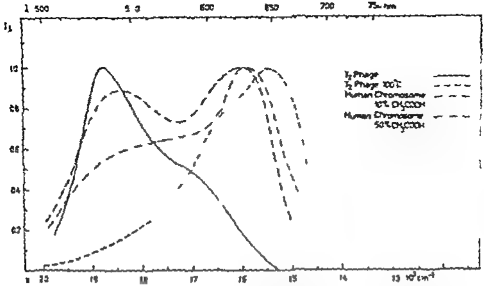


Fig 33 Emission spectra of AO bound to T_2-phages before and after heating to 100 C, and to human chromosomes prepared in 10 and 50 per cent acetic acid. Standard staining

squashing in 10 per cent acetic acid show random coil as well as helix regions the helix regions are further diminished if 50 per cent acetic acid is used In the following compilation the amount of helix and random coil regions has been calculated for the four samples of Fig 33 using the α value calculated from their uncorrected fluorescence intensities at 590 and 530 nm The standards used for pure helix regions and pure coil regions in nucleoproteins were $\alpha_h = 0\ 140$ and $\alpha_s = 3\ 31$ $C = 39\ 1$ (see Chapter 4)

Thus quantitative approximation indicates that the DNA of this T phage sample shows only about half the amount of helix regions compared with the DNA in the intracellular DNP complex of human leucocytes ($\alpha = 0\ 140$) which was used as the helix reference Heating the phages causes an almost

	α	Helix region %	Random coil region %
T_2 phage	0 225	48	52
T_2 phage 100	1 86	2	98
Human chromosome 10 % acet acid	0 390	23	77
Human chromosome 50 % acet acid	0 501	17	83

total loss of helix structure In the case of human chromosomes, more than two thirds of the helix structures are destroyed by treatment in 10 per cent acetic acid and even more when 50 per cent acetic is applied This very common method for obtaining chromosomes is thus useless for an investigation of the chromosomal nucleic acid structure

2 Quantitative determination of AO binding groups in nucleoproteins

As explained in Chapter 4 the amount of dye binding nucleic acid groups, as represented by the negatively charged phosphate groups, can be estimated in a single nucleoprotein as well as in a mixture of two nucleoproteins Provided there is no interference with the concomitant protein, the bound AO will serve as a measure for all dye binding phosphate groups and hence for the total amount of the nucleic acid constituting the nucleoproteins If, however, the AO binding nucleic acid groups are partially blocked, the AO uptake will instead correspond to the number of 'free' unblocked phosphate groups As already discussed the number of free dye binding phosphate groups may be determined by using the isolated nucleoprotein AO complexes as a reference, there being no demonstrable interaction between protein and nucleic acid when the protein amino groups were blocked with acetic anhydride at 20° The determination of free dye binding phosphate groups, however, is applicable only to the DNP complex not to the RNP complex which exhibits large differences in the AO emission spectrum with increasing and decreasing protein nucleic acid interaction

As will be shown AO stained intracellular nucleoproteins displayed large changes in the amount of free phosphate groups in DNP but not in RNP, when the standard acetylation procedure was applied Intracellular RNP, whenever it is present in sufficient amounts for an emission spectrum to be properly recorded shows an AO aggregation as measured by the α value α 2 9 that is close to the dye aggregation of the isolated RNP AO complex α 3 31, Since α in the RNP AO complex changes with increasing and decreasing protein nucleic acid interaction it would seem that none, or only a minor percentage of the phosphate groups are blocked in intracellular RNP In addition the α of intracellular RNP AO complexes may have been underestimated and that of isolated RNP AO complexes overestimated in the former case the possible presence of cytoplasmic DNA (Nass and Nass 1963 Brachet and Quertier 1964 Sager and Ishida, 1963) might give lower values of α for the cytoplasmic RNP AO complex in the latter the RNA structure may be affected by the use of deoxycholate in the isolation procedure In subsequent calculations it was therefore assumed that after acetylation with

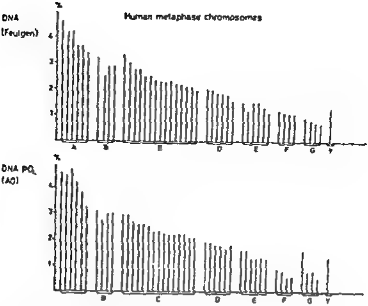


Fig 34 Relative content of DNA (Feulgen staining) and of AO binding DNA PO_4 groups (AO-staining) of individual human chromosomes in the metaphase Chromosomes paired after their morphological aspect and arranged in main groups A B C Y

acetic acid anhydride all phosphate groups of the intracellular RNP are able to bind AO

As explained in Chapter 4 the amount of dye binding free phosphate groups in intracellular DNP and RNP can be determined from equations (24)—(29) assuming that all phosphate groups of intracellular RNP like those of the isolated RNP are available for the dye

A very simple case is the one where only DNP has to be considered either because no RNP was ever present or because this has been extracted by RNase acids or buffer solutions This is exemplified with human metaphase chromosomes isolated by a squash technique in acetic acid (Fig 34) Since all the ribosomal particles seen in the unsquashed cell had completely disappeared after squashing in acetic acid and AO staining the chromosomes could be treated as pure DNP particles There was good agreement on the average between the relative amounts of free phosphate groups of the metaphase chromosomes and the relative amount of DNA as estimated by the Feulgen technique if one disregards small differences for the smallest chromosomes Some very interesting aspects would be presented by these differences if they are significant (see next section) however too little material has been investigated so far

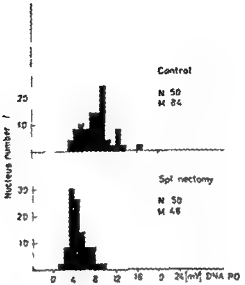


Fig 35 Frequency distribution of AO binding DNA PO_4 groups (in mV) in the nuclei of the deciduoma of pseudo-pregnant rats before and after splenectomy Standard staining

Another case studied was the DNP in nuclei of decidua cells from traumatized uterus mucosa of pseudo pregnant rats When the traumatization which causes the formation of the decidua cells is accompanied by extirpation of the spleen the decidua cells are found to have only one half of the otherwise diploid chromosome set (Rigler and Rosenkranz, 1958, Rosenkranz, and Rigler 1958 Bouvier *et al* 1960) Further when saline extract of rat, cattle or horse spleen is injected into the splenectomized rat this haploidization is prevented The decidua cells of splenectomized rats, besides having only the haploid chromosome set, have only half the amount of free DNA phosphate groups Fig 35) This indicates that in both the diploid and the haploid chromosome set the same relative amount of phosphate groups was available for dye binding *i e* the haploidization did not alter the amount of free dye binding phosphate groups per nucleotide unit

A third example concerns the DNP of the reduplicating macronucleus of the protozoon *Euplotes* This reduplication process begins at both ends of the macronucleus causing the formation of so called reorganization bands which move from either end of the macronucleus towards the middle During this process the DNA Gall, 1959) and the protein content (Ringertz and Hoskins 1964 are both doubled, and so is the amount of free dye binding phosphate groups in the reorganization bands (in this case expressed by the fluorescence intensity at 530 nm Fig 36)

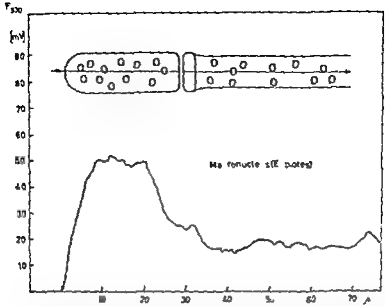


Fig 36 Distribution of the fluorescence intensity at 530 nm (F_{530}) along the macronucleus of *Euplotes* Scanning along the longitudinal axis of the macronucleus with a slit diaphragm (30 μ × 3 μ) vertical to the scanning direction and automatic registration of the fluorescence intensity Original curve DNA reduplication commencing at the nucl ar end (reduplication band) Standard staining

The next possibility considered is the determination of dye binding phosphate groups in intracellular DNP and RNP The example given is the relative and absolute amount of dye binding phosphate groups of DNP and RNP in a mouse fibroblast culture These amounts of DNA PO_4 and RNA PO_4 groups have been calculated for each mouse fibroblast cell from their a value a_{DNP} and a_{RNP} should ideally be represented by the values of a for the intracellular RNP AO and the intracellular DNP AO complex of the mouse fibroblast cell Since an accurate determination of a_{RNP} in the cell is not possible because of the simultaneous presence of DNP the value of the isolated RNP AO complex acetylated at 20 C and not corrected for protein fluorescence ($a_{RNP} = 3\ 31$) was used for a for the pure RNP AO complex The value of the intracellular DNP AO complex of mouse leucocytes isolated from lymph nodes was taken to be the a for the pure DNP AO complex ($a_{DNP} = 0\ 154$) The DNP of mouse leucocytes perhaps best resembles the DNP of mouse fibroblast cells (as shown in the next section) and can be regarded as almost free of RNA A determination of a of the mouse fibroblast DNP after enzymatic extraction of RNA did not appear to give as accurate

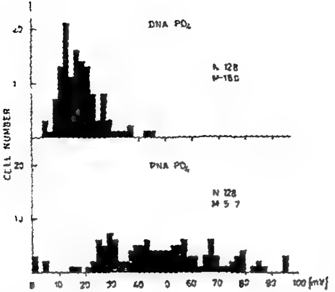


Fig. 37 Frequency distribution of AO binding free DNA PO_4 and RNA PO_4 groups (in mV) in cells of a mouse fibroblast culture (L-929) after growing for 48 hours. Standard staining. Biochemical determination of DNA and RNA in the same culture

results since the extraction procedure affected the DNA structure as well. C is then calculated from equation (25) as 35.1. The relative amounts of DNP PO_4 and RNP PO_4 have been determined from equations (24), (26) and (27) and their frequency distributions plotted in Fig. 37. The mean for the DNA PO_4 distribution is 18.0 mV and for the RNP-PO_4 distribution 50.7 mV. To calculate the absolute amount of free DNA phosphate groups the relative amounts are divided by the fluorescence coefficient of DNP at 530 nm, using equation (28) in Chapter 4. The fluorescence coefficient of the mouse lymphocyte DNP has been calculated according to equation (20a) from the measured α value. If $\alpha = 0.154$ then $F_{530}/10^{-15}$ mole P $= f_{530}^{DNP} = 322$ mV/10^{-15} mole P. Dividing the relative amount of DNP PO_4 in mV by the fluorescence coefficient gives the amount of free dye binding phosphate groups as an average of 55.8×10^{-15} mole PO_4. Direct biochemical determination of the DNA phosphorus of the same culture gives 74.0×10^{-15} mole P per cell which means that only approximately 75 per cent of the total nucleic phosphorus or phosphate groups are free to bind AO (see Table VII).

The ratio of free dye binding RNP PO_4 groups to free DNA PO_4 is 2.8. This has to be multiplied by 0.75 to obtain the ratio of the total RNA PO_4 groups to the total DNP PO_4 groups since the free phosphate groups represent

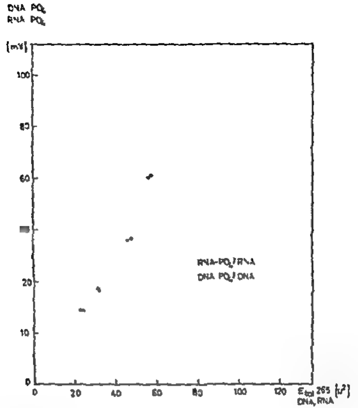


Fig 38 Relation of the relative DNA PO_4 and the relative RNA PO_4 content (in mV) of mouse fibroblast cells to their relative DNA and RNA content expressed by the E_{tot} 265 (corrected for unspecific light loss and protein absorption)

only 75 per cent of all phosphate groups The result 2.1 is very close to the RNA phosphorus/DNA phosphorus ratio obtained by biochemical determination on the same culture i.e. 1.85

This calculation tacitly assumes that the RNA PO_4 groups of the intracellular RNP are available for the dye as discussed earlier In addition to simply comparing the α values of intracellular RNP AO and isolated RNP AO complexes, this was tested by comparing the total amount of DNA PO_4 and RNA PO_4 groups as calculated in the manner just described with the DNA and RNA content as determined by a combined UV and Feulgen technique (see Chapter I) Technically, this was arranged so that UV measurements were followed by AO staining and AO staining by Feulgen staining on the same cells

If this type of calculation is correct, and assuming that all RNA phosphate groups are free, then plots of RNA PO_4 against RNA and of DNA PO_4 against DNA should obey the same function. As shown in Fig. 38, this is in fact the case.

A further example, the calculation of free DNA and RNA phosphate groups in the DNP and RNP complexes of human lymphocytes, stimulated to growth by phytohemagglutinin (PHA), is given in the next section of this chapter. The relative amounts of free DNA and RNA phosphate groups were determined at varying intervals after the addition of PHA to the lymphocyte culture. The frequency distributions are shown in Fig. 41. a_{RNP} was again taken as 3.31, while the a of polymorphonuclear leucocytes from the same culture was taken for a_{DNP}. Their a was consistently the lowest of all leucocytes (0.159) in this culture, moreover they never produced RNA, even when the lymphocytes showed an abundant RNA synthesis. It was therefore assumed that polymorphonuclear leucocytes neither contain nor produce RNA; this is supported by the data of Thorell (1947) and by the observation that practically no incorporation of P^{32} or C^{14} could be found in the cytoplasma of mature granulocytes (Lajtha *et al.*, 1954). The polymorphonuclear leucocytes do however display the same alteration in their DNP as the lymphocytes upon the administration of PHA and are therefore regarded as an ideal reference system for the determination of a_{DNP} which, as will be shown, is dependent upon the functional stage of the DNP complex.

Thus, for the calculations behind Fig. 41, a_{DNP} was 0.159 and a_{RNP} 3.31, C was calculated to be 33.9. The highest RNA PO_4 /DNA PO_4 ratio was computed as 1.8, the average as 1.0 after 72 hours growth in the *in vitro* culture. Since only about 41 per cent of the phosphate groups in this culture are free for dye binding (see Table VII), the total RNA PO_4/DNA PO_4 ratio works out at about 0.4 to 0.7. This is similar to the values found for RNA P/DNA P in growing lymphocytes and lymphoblasts of bone marrow and in lymphatic leukemias (Davidson *et al.*, 1951).

3. *Sensitivity, reproducibility and limits of the method*

One of the big advantages of this method is its extreme sensitivity. The lowest amounts of nucleic acids measurable at a signal to noise ratio of 20 : 1 was found to be approximately 5×10^{-18} mole DNA PO_4 and 5×10^{-17} mole RNA PO_4. This would correspond to about 10 T_2 phage particles or about 3—4000 tobacco mosaic virus particles. Yet this is not the limit. By using more sensitive photomultipliers, exitation sources of higher brightness and filters instead of monochromators, the sensitivity of the measurement could

be heightened by a factor of at least 200. This suggests that a single particle of phages of the T type could be measured without any difficulty.

At all events this method should be useful for detecting small amounts of cytoplasmic DNA, as postulated by many authors, and give at the same time information about its structural conformation.

The mean fluorescence intensity of 10 lymphocytes was determined after two successive stainings of cells of the same culture to test the reproducibility of the method. The difference between the mean fluorescence intensities was found to be not more than 4 per cent (average of 5 experiments).

The quantitative determination of nucleic acids is impeded if the concentration of the nucleic acid AO complex or the layer thickness is so high that not every AO molecule can be excited because the exciting radiation has been absorbed to a major extent and the emitted radiation of an exited molecule does not reach the photomultiplier but is reabsorbed by the AO itself. This is quite common for a number of fluorescent dyes owing to their overlapping absorption and emission spectra. The influence of the absorption of the exciting and the reabsorption of the emitted radiation has been discussed in Chapter 2, where equation (8) gives the relation between the absorption of the exciting and reabsorption of the emitted radiation on the one hand and the fluorescence intensity on the other for an object excited as in case A, Fig. 5. However the fluorescence intensity is a direct measure of the amount of excited substance only if the absorption and reabsorption are very slight. Equation (8) can then be replaced by equation (11), which gives the simple relationship between fluorescence intensity and the concentration and layer thickness of the excited substance that makes the fluorescence measurement so simple and easy. The linear relation between the fluorescence intensity I_λ and the concentration expressed by the absorption coefficient k_λ is shown in Fig. 6. If a certain absorption and reabsorption are exceeded, however, I_λ does not follow the linear function of Fig. 6 (case b) but one of the exponential functions (case a). The fluorescence intensity then increases exponentially with the concentration of the excited substance, reaches a plateau and decreases if the concentration or the layer thickness is further increased. In this case the measured fluorescence intensity is no longer proportional to the total amount of excited substances.

To give an idea of how much the emitted fluorescence intensity deviates from the linear relation to concentration and layer thickness, the fluorescence intensity at 530 nm (I_λ) of AO solutions of different concentrations has been calculated from equation (8) in Chapter 2 for a given extinction coefficient of the excitation wavelength 365 nm and of the emission wavelength 530 nm (where a reabsorption of the emitted radiation has to be taken into account) at a given layer thickness. This was compared with the fluorescence intensity

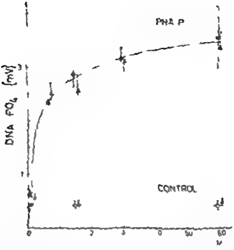


Fig 40 Increase of the AO binding free DNA PO$_4$ groups in mononuclear (●) and poly nuclear (▲) human leucocytes in relation to the time after the addition of phytohemagglutinin (PHA) to the *in vitro* culture Open symbols are controls Mean values and 95 per cent confidence intervals (n = 10) Standard staining (From Killander and Rigler Jr 1965)

(Killander and Rigler Jr 1965) which is the expression of a sudden increase of dye binding phosphate groups in the DNP The relative amount of dye-binding phosphate groups increases vigorously 5 minutes after the addition of PHA to the lymphocyte culture and reaches a plateau after 60 minutes (Fig 40) This increase in the reactive phosphate groups which is likewise found in polynuclear leucocytes is however not accompanied by a rise in the DNA content In fact the DNA content measured by the UV absorption and the Feulgen reaction remains constant throughout the continuous increase of the dye binding phosphate groups This increase in dye binding phosphate groups reflects the liberation of anionic phosphate charges within the DNP molecule

The amount of the dye binding DNA phosphate groups is maintained constantly on a plateau value reached 60 minutes after the addition of PHA, while the initially insignificant amount of dye binding RNA phosphate groups begins gradually to increase (Fig 41) 48 hours later a second population of DNA phosphate groups appears and becomes especially marked after 72 hours The mean of this population is twice as high as that of the original one UV and Feulgen measurements have shown the DNA synthesis to be initiated at the same time the increase in the dye binding DNA phosphate groups thus represents a synthesis and not a liberation of phosphate groups as in the initial stage of the stimulation with PHA This interpretation is also supported by the doubling in the number of dye binding DNA phosphate groups in the meta-

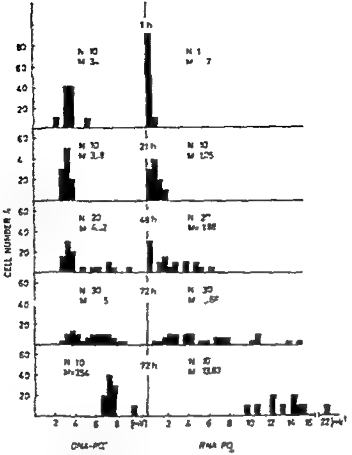


Fig. 41 Frequency distribution of AO-binding "free" DNA PO⁻ and RNA PO⁻ groups (in mV) in human mononuclear leucocytes 1, 21, 48 and 72 hours after the addition of phytohemagglutinin to the culture *in vitro*. The bottom two frequency diagrams show the distribution of DNA PO⁻ and RNA PO⁻ in metaphase plates (3h Colcemide). Standard staining

phase (bottom diagram in Fig. 41). The dye binding RNA phosphate groups increase continuously with the age of the culture.

A striking phenomenon of this investigation was the almost immediate liberation of the reactive DNA phosphate groups following the addition of PHA to the lymphocyte culture. Similar observations have been made by Cooper and Rubin (1965) demonstrating an increased incorporation of H^3-uridine into lymphocytes within 60 minutes of stimulation with PHA. Within the same time interval a decrease in basic charges of nuclear histones was observed as revealed by Fast Green and ammoniacal silver staining after antigen stimulation of mouse thymocytes and lymphocytes (Black and

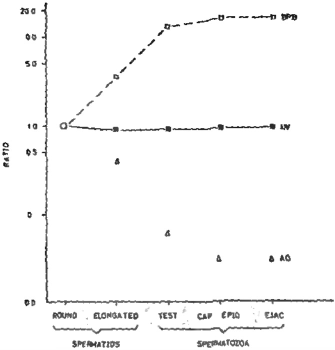


Fig 42 The decrease of acridine orange (AO) binding DNA PO groups (△) together with the increase of bromphenol blue (BPB) binding histone NH groups (□) of the DNP complex during spermiogenesis. Changes given as the ratio of the values obtained for the immature haploid round spermatid (○) The reference given for the relative amount of the total DNA PO_4 groups is the UV absorption (■) of the DNP complex in all stages of sperm maturation (round and elongated spermatids testicular caput epididymal and ejaculate spermatozoa) (From Gledhill Gledhill Rigler Jr and Ringertz 1966)

Ansley, 1965) A connection between those two observations and the liberation of DNA phosphate groups after the stimulation with PHA seems to be likely

Since a synthesis of RNA phosphate groups was never observed without a preceeding liberation of DNA phosphate groups this phenomenon was assumed to be one prerequisite of the RNA synthesis However it may not be the only one since in polymorphonuclear leucocytes showing the same liberation of DNA phosphate groups as lymphocytes, no succeeding RNA synthesis was observed

The opposite behaviour in the dye binding of their DNA phosphate groups was shown by cells passing from genetically active into genetically inactive stages (Gledhill *et al* 1966) During sperm maturation a dramatic reduction of the dye binding phosphate groups was found which began at the stage of the round spermatid and was completed when the caput spermatid had

developed (see Table VII) At this stage the dye binding DNA phosphate groups amounted to only about 4 per cent of the value found for round spermatids. During this reduction of dye binding DNA phosphate groups which was accompanied by an almost equivalent increase of basic groups of the histone component (brompherol blue binding substances the DNA content was found to be constant (Fig 42 Thus the reduction of the dye binding DNA phosphate groups in the final stage of sperm maturation has to be considered as a neutralization, probably caused by the increase in the basic histone charges and constitutes no loss of DNA Against this the decrease in dye binding DNA phosphate groups found in the first stage of the sperm maturation leading from the diploid or tetraploid spermatocytes to the haploid round spermatides reflects the partitioning of the total DNA by meiosis a proportional decrease in dye binding DNA phosphate groups and in total DNA is found

In general the sperm maturation is the exact opposite of the lymphocyte stimulation with PHA Lymphocyte stimulation starts with a liberation of dye binding DNA phosphate groups which is followed by a synthesis of new dye binding phosphate groups and a mitotic division Sperm maturation begins with a meiotic division and a partition of dye binding DNA phosphate groups which are finally neutralized

If the genetic activity of the cells as measured by their ability to synthesize RNA greatly enhanced in PHA stimulated lymphocytes and completely abolished during the sperm maturation Monesi 1964 1965 then an apparent correlation exists between the liberation of reactive dye binding DNA phosphate groups and a genetic activation and between a neutralization of reactive dye binding phosphate groups and a genetic inactivation. The phenomenon of phosphate group liberation seems to be a common one stimulation of human lymphocyte cultures by specific antigens tetanus toxoid, tuberculine) or by streptolysine likewise leads to a liberation of DNA phosphate groups and to a genetic activation Soren preliminary observations

Several checks were made showing that the diffusion of the AO to the intracellular DNP was not influenced by an altered permeability of the cell membrane which could cause the differences in dye binding Neither an increase of the AO concentration of the staining solution nor an increase in the staining or in the rediffusion time altered the relative difference between the dye uptake of the unstimulated and the stimulated DNP The same was true if the acetylation procedure increasing the dye binding of the DNP by a factor of 4 was omitted or the cells had been stained at neutral pH Likewise an altered protein fluorescence or an increased AO binding of the protein component, which might possibly falsify the result could be excluded

TABLE VII. Fluorescence intensity at 530 nm, α value and [a] free AO binding DNA-PO$_4$

			Fluorescence intensity at 530 nm (F_{530}) [mV] Mean ± S.E.	α (F_{530}/F_{640}) Mean ± S.E.
Man	Mononuclear leucocytes			
		mean	1.[illegible]6 ± 0.24	0.203 ± 0.023
		min	0.40 ± 0.01	0.26[illegible] ± 0.017
		max	[illegible].32 ± 0.40	0.144 ± 0.003
	1 h PHA	mean	3.02 ± 0.34	0.1[illegible]4 ± 0.00[illegible]
		max	8.[illegible]7 ± 0.33	0.180 ± 0.006
	Polymorphonuclear leucocytes			
		mean	1.[illegible]1 ± 0.20	0.1[illegible]6 ± 0.006
		min	0.47 ± 0.02	0.2[illegible] ± 0.010
		max	7.[illegible]1 ± 0.33	0.140 ± 0.003
	1 h PHA	mean	3.[illegible] ± 0.7[illegible]	0.16[illegible] ± 0.007
		max	9.[illegible] ± 0.[illegible]	0.142 ± 0.00[illegible]
	Spermatozoa		0.074 ± 0.00[illegible]	0.31[illegible] ± 0.04[illegible]
Mouse	Mononuclear leucocytes		[illegible]9 ± 0.[illegible]3	0.1[illegible]4 ± 0.00[illegible]
	Polymorphonuclear leucocytes		4.22 ± 1.10	0.1[illegible]8 ± 0.01[illegible]
	[illegible] blast cells (L 42[illegible])		13.[illegible] ± 0.6[illegible]	0.331 ± 0.008
Bull	Spermatocytes		11.9[illegible] ± 0.72	0.20[illegible] ± 0.010
	Round spermatids		[illegible].71 ± 0.16	0.21[illegible] ± 0.006
	Elongated spermatids		1.4[illegible] ± 0.16	0.1[illegible]7 ± 0.009
	Testicular spermatozoa		0.210 ± 0.01[illegible]	0.2[illegible] ± 0.042
	Caput spermatozoa		0.10[illegible] ± 0.013	0.27[illegible] ± 0.02[illegible]
	Ejaculated spermatozoa		0.10[illegible] ± 0.01	0.272 ± 0.0[illegible]

[a] Determined by ultraviolet absorption at 26[illegible], 280 and [illegible] nm (see materials and methods)

The quantitative changes of the dye-binding phosphate groups of DNP during the different stages of cell activity have been compiled in Table VII, which shows the fluorescence intensity at 530 nm, the α value, and the reciprocal fluorescence coefficients calculated from the α values after equation (20a) of Chapter [illegible]. In addition, it shows the absolute amount of dye binding DNA phosphate groups calculated as discussed in the first section of this

[illegible] of DNP of different [illegible] and under different [illegible] conditions

No. of cells	Response fluorescence coefficient [illegible]	"Free" DNA PO groups [illegible]	Total DNA PO groups [illegible]	DNA-PO / DNA-PO
[illegible]	4.1	[illegible]	[illegible]	[illegible]
10	3.7	[illegible]	[illegible]	[illegible]
10	2.8	[illegible]	[illegible]	[illegible]
40	3.5	[illegible]	[illegible]	[illegible]
10	2.8	[illegible]	[illegible]	[illegible]
[illegible]	4.1	[illegible]	[illegible]	[illegible]
10	[illegible]	[illegible]	[illegible]	
1	2.8	[illegible]	[illegible]	[illegible]
[illegible]	3.5	11.8	[illegible]	[illegible]
1	2.8	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	1	[illegible]
[illegible]	3.1	15.3	[illegible]	[illegible]
10	3.2	13.5	[illegible]	[illegible]
128	3.1	[illegible]	[illegible]	[illegible]
[illegible]	4.1	44.5	[illegible]	[illegible]
43	4.1	[illegible]	4.8	[illegible]
10	4.1	6.11	14.5	[illegible]
10	[illegible]	1.18	14.2	[illegible]
40	[illegible]	[illegible]	14.3	[illegible]
[illegible]	[illegible]	[illegible]	14.2	[illegible]

Determined by diphenylamine after [illegible] acid extraction by TCA

chapter and the total amount of the DNA phosphate groups determined by UV spectrophotometry (see Chapter 1) as well as the ratio of reactive dye-binding to total phosphate groups (DNA PO/DNA PO). The values of the lymphocyte experiments refer to one hour PHA stimulation; the controls had been incubated for the same time in a medium without PHA. For the calculation of the fluorescence coefficients for the lymphocytes the [illegible] values

for the polymorphonuclear leucocytes from the same culture were taken, the DNP of the latter showed exactly the same changes as that of lymphocytes and can be considered to contain no or negligible amounts of RNA This is also indicated by the inability of polymorphonuclear leucocytes to synthesize RNA In the case of the sperm maturation, the α of the elongated spermatids has been used to calculate fluorescence coefficients for the round spermatids and spermatocytes The fluorescence coefficients for the remaining cell species have been calculated from their respective α values

As can be seen in Table VII the portion of the reactive DNA phosphate groups amounts to about 25 per cent of the total phosphate groups in lymphocytes as well as in polymorphonuclear leucocytes The leucocytes of all five donors investigated on the average exhibited the same relative amount of dye binding phosphate groups although big individual variations were found ranging from approximately 10 to 80 per cent of the total phosphate groups Even within the same donor variations between 20 and 80 per cent of liberated phosphate groups were found which may reflect different degrees of cell activity After one hour s action of PHA, the number of liberated phosphate groups is approximately doubled (inclusive of all negative experiments) The action of PHA however showed big fluctuations, ranging from no effect to complete liberation of all phosphate groups, it was to a certain extent dependent on the culture conditions but even more so on the PHA preparation used Preliminary results, indicating a functional relationship between the DNA phosphate groups liberated one hour after addition of PHA and the RNA phosphate groups synthesized 48 hours later, suggest an intrinsic connection between the liberated phosphate groups and the activity of the genome If the liberated DNA phosphate groups increased by a factor of 2 then the RNA-phosphate groups synthesized 48 hours later increased approximately by a factor of 4 During the synthesis of DNA phosphate groups between 48 and 72 hours after PHA administration the original amount of DNA phosphate groups was always doubled before mitosis regardless of how many DNA-phosphate groups had been liberated 60 minutes after PHA addition It seems as if once liberated the amount of DNA phosphate groups is always kept constant it is consequently doubled before mitosis The nucleic acid synthesis may proceed on different levels

Mononuclear and polymorphonuclear leucocytes from mouse lymph nodes showed 50—70 per cent liberated phosphate groups without any stimulation of PHA Interestingly the number of liberated DNA phosphate groups could not be increased by addition of PHA Also in the mouse fibroblast cells of Fig 37, about 75 per cent of the total DNA phosphates had already been liberated

During bull sperm maturation the DNA phosphate groups which are almost

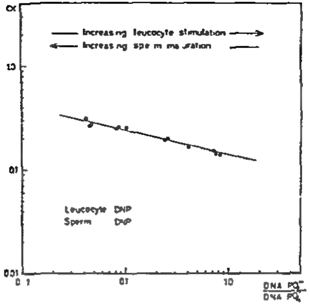


Fig 43 α values of the DNP AO complexes of bull spermatids and spermatozoa of different maturation stages as well as of human spermatozoa and human leucocytes of different degrees of stimulation in relation to their relative amounts of AO binding free DNA PO groups

completely liberated during the stage of meiosis decrease to only 4 per cent if the maturation process is completed The same small amount of liberated DNA phosphate groups is found in human spermatozoa

An analysis of the α values of different stages of cell stimulation and genetic activity reveals significant differences Moreover if the α values are plotted versus the liberated DNA phosphate groups of the same cells expressed as a fraction of the total DNA phospate groups (Fig 43) a functional dependence between these two values can be found α varies inversely to the amount of liberated DNA phosphate groups The increase of α with decreasing number of liberated phosphate groups cannot be explained by a stronger binding of the histones to the DNA since this could in theory only lead to an increased dye binding in the monomer molecular form as in the case of the RNP (see Chapter 4) and hence to a decrease in α The increase of α with increasing neutralization of the DNA phosphate groups can only be explained by a change in the chain organization of the DNA This means that with increasing liberation of DNA phosphates the DNA helix is more highly ordered as indicated by a decrease of α This decrease of α is virtually caused by an enlarged distance between the bound AO molecules (as discussed earlier)

which accompanies the higher degree of nucleic acid chain organization The greater distance between AO molecules may be due to an increased distance of the binding sites on the DNA i e the anionic charged phosphate groups as well as to an impaired intercalation of the AO molecules between the nucleotide bases This would be best explained by an extension of the DNA molecule accompanied by a higher ordering of the DNA helix

Final interpretation

The view cited above is supported by the observations of Zubav and Doty (1959) investigating the hydrodynamic properties of DNA and DNP By measurement of the extinction angle and the birefringence of flow, the effective length of the hydrodynamically equivalent ellipsoid of DNA was found to be about double that of DNP Moreover the results indicate that DNP may not have as many base pairs perpendicular to its direction of alignment as does DNA Similar observations were made by Giannoni and Peacocke (1963) by measuring the molecular weight sedimentation and intrinsic viscosity the DNP molecule behaved like a moderately stiff coil in contrast to the stiff coil of DNA These results demonstrate that the DNA molecule is more extended than the DNP molecule and has a higher degree of chain organization The reason for this behaviour is the mutual repulsion of the unscreened equally charged phosphate groups This repulsion does not only cause a lengthening and a higher organization of the DNA molecule but diminishes at the same time the stability of the DNA helix. DNA exhibits a higher thermolability than DNP the melting temperature T_m is found to be at lower values than that of DNP (Lee *et al* 1963 Zubay and Doty 1959 The thermolability of the DNA molecule likewise increases if the ionic strength of the solvent is diminished (Peacocke and Walker 1962, Colvill and Jordan 1963 Kotin 1963) In both cases the instability of the DNA helix is caused by the decreasing neutralization of the anionic phosphate groups b counter ions

These findings which are the result of differences in the dye binding capacity of DNP in different functional stages prompt the following conclusions A genetic activation of the cell by the stimulation of a variety of agents (PHA antigens bacterial toxins) is accompanied by an immediate liberation of dye-binding phosphate groups of the DNP complex This causes the DNA molecule to elongate and to assume a higher degree of structural organization, i e a higher degree of helix formation At the same time this implicates an increased lability of the helix structure by the weakening of the hydrogen bonds as a result of the mutual repulsion of the unscreened phosphate groups A genetic inactivation as seen during the maturation process of sperms results in a neutralization of dye binding phosphate groups of the DNP complex leading

to a contraction of the DNA molecule with a simultaneous decrease of the structural organization i.e. to a less perfect DNA helix. These molecular changes increase at the same time the stability of the DNA molecule.

From the biological point of view this activation of the DNA molecule by the liberation of reactive DNA phosphate groups should make the molecule especially suitable for its function as a template i.e. an increase in the order of helix regions enhances the enzymatic synthesis of RNA (Wood and Berg 1964, Hayashi *et al.* 1963). The increased lability of the helix structure connected with the liberation of reactive phosphate groups should facilitate the strand separation during the DNA replication following a semiconservative (Taylor 1963) or a conservative mechanism (Cavalieri and Rosenberg 1963).

On the other hand neutralization of the reactive phosphate groups leading to a diminished structural organization of the DNA helix and to a higher stability of the DNA molecule, should favour the task of the reproductive cells in preserving the genetic information unaltered by outside influences.

In fact observations on nucleic acids extracted from growing bacteria (Lark 1963, Rolfe 1963) and human cell cultures (Rosenberg and Cavalieri 1964) indicate the presence of an "activated" DNA. In these experiments the appearance of a metastable DNA with a boyant density characteristic for denatured DNA was observed before the beginning of the RNA and DNA synthesis. The appearance of this heavy satellite band is however dependent on the isolation conditions and is only found if the cells are lyzed by high temperature, deproteinizing agents and high shearing forces. The denaturation of this DNA fraction is assumed to be latent *in vivo*, the actual collapse of the molecular structure is brought about by certain conditions during isolation.

A DNA with unscreened phosphate groups should possess exactly these properties: an identity between the DNA activated by phosphate group liberation and the metastable DNA described by Rosenberg and Cavalieri seems likely. This identity is further reinforced by the observation that as in Rosenberg and Cavalieri's system a functional dependence seems to exist between the amount of "activated" DNA (liberated phosphate groups) and the amount of RNA later synthesized.

The increase in basic protein groups accompanying the neutralization of the dye binding phosphate groups during sperm maturation suggests an important role for the concomitant protein in the function of the DNA template.

Summary

The practical application of the AO technique for the investigation of intracellular nucleic acids and nucleoproteins is discussed regarding the spectral analysis, the determination of helix and coil regions in single nucleoproteins and the

and emission spectra of the RNP AO complex the absorption at 500 nm and the emission at 530 nm become greatly accentuated, this is not the case when the amino groups are blocked with acetic acid anhydride This suggests that in the former case there is a dye binding in the monomer molecular form at the expense of the associated, the associated form of dye binding is obstructed by the increased protein nucleic acid interaction No such change is displayed by the DNP AO spectra on increasing protein nucleic acid interaction since AO binds to isolated DNA already in the monomer form Quantitative studies have been performed at acid pH (pH 4) in order to avoid binding of AO to the anionic groups of the histones and ribosomal proteins DNP and RNP behave like the free nucleic acids in binding AO when the amino groups of the proteins are blocked with acetic acid anhydride Under these conditions the amount of helix and coil regions can be determined in nucleic acids of nucleoproteins as in free nucleic acids, from the size of a for their AO complex In the case of a mixture of two nucleoproteins or nucleic acids of differing chain order a can be used to determine their ratio as well as their relative and absolute amounts If the binding form of AO to nucleic acids is not altered by the protein nucleic acid interaction as indicated in the case of DNA the amount of free dye binding sites (phosphate groups) on the nucleic acid can also be determined The equations for these calculations are derived and examples given

The AO binding to the intracellular DNP and RNP of fibroblast cells is dependent on the ionic strength of the buffer used for the staining procedure as well as on the dye concentration the staining time and the rediffusion time (time during which the unbound AO is allowed to diffuse out of the cell into pure buffer solution) The amount of dye per nucleotide unit (AO P ratio) as expressed by the ratio a, increases with increasing dye concentration and staining time and decreases with increasing ionic strength of the buffer and rediffusion time The influence of an unspecific AO binding of cell proteins at different pH and of different fixation methods as well as the kinetics of the acetylation procedure are studied The procedure for a reproducible and quantitative staining of intracellular nucleoproteins is given

Several biological samples have been studied *e g* mouse fibroblast cells, leucocytes and spermatozoa of various origins bacteriophages, isolated cell nuclei and human metaphase chromosomes The amount of helix and coil regions of DNA in the intracellular DNP complex has been calculated, as has the amount of free dye binding phosphate groups in DNP and in RNP The smallest amount of nucleic acid detectable with the measuring device used corresponds to about 5×10^{-18} mole DNA PO_4 and 5×10^{-17} mole

RNP PO_4 The reproducibility of the method is discussed together with certain limitations caused by the strong absorption of the exciting radiation and the reabsorption of the fluorescence in thick layers

The last example given [illegible] the application of AO binding to determine the amount of dye binding phosphate groups and the degree of ordered structure of DNA in the DNP complex in different stages of cell activity Increasing genetic activity, as deduced by the ability of cells to produce ribosomal RNA (studied in human lymphocytes stimulated to growth with phytohemagglutinin) is combined with a marked liberation of dye binding DNA phosphate groups of the DNP complex, indicating a partial or complete dissociation of the DNP complex into DNA and histones At the same time the degree of ordered structure of the DNA is increased Decreasing genetic activity (studied in bull sperms during sperm maturation) is connected with a reduction of the dye binding phosphate groups of the DNP complex to about 4 per cent of the original value (haploid round spermatids) suggesting a complete association of DNA and the concomitant proteins The structural order of the DNA decreases at the same time A functional dependence was found between the degree of structural organization of DNA expressed by the ratio [illegible] and the relative amount of liberated phosphate groups The degree of structural organization of DNA in the DNP complex increases with increasing liberation of dye binding phosphate groups Synthesis of ribosomal RNA was never found without previous liberation of DNA phosphate groups, and preliminary data indicate a connection between the amount of liberated phosphate groups and the amount of RNA synthesized the importance of the liberation of phosphate groups of the DNP complex for the template function of DNA is therefore discussed

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OCULAR AND ORBITAL VEGETATIVE NERVES

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CONTENTS

INTRODUCTION

Although the classical techniques for demonstrating nerve fibres have been found useful in the investigation of the general morphology and topography of many neuronal systems, they are incapable of clearly distinguishing the adrenergic and cholinergic divisions of the vegetative nervous system unequivocally and in detail. Consequently, reliable fundamental data are lacking in the discussion of problems concerning the peripheral vegetative innervation. The epoch-making fluorescence technique of Falck and Hillarp for determining the cellular localization of catecholamines and 5-hydroxytryptamine has, however, made the above distinction possible. In recent modifications, the Koelle technique for cholinesterases is most probably also a sufficiently specific method for demonstrating cholinergic nerves, and as such it is presumably the best technique available.

The present study was designed against the background of the mentioned achievements. Ocular structures have long been important as model systems in the investigations of many of the fundamental aspects of neurons as well as of other tissue components. The ready accessibility of the eye to experimental manipulations and inspection, the avascularity of the cornea, the regular organization of the retina, and the unusual possibilities of selective denervation are some of the important and unique features that make the various ocular tissues useful as tools for model studies pertaining to the whole organism. Moreover, observations made in any study on the autonomic nerves has obvious connections with the physiology and pharmacology of autonomic substances and drugs, whose actions and clinical applications have previously been thoroughly studied in the eye.

The purpose of the present investigation was to chart the normal distribution of adrenergic and cholinesterase-containing nerve fibres in the eye and to study the relationship between peripheral adrenergic and cholinergic fibres. Pilot studies revealed that the distribution of adrenergic fibres varied widely from species to species. Detailed know-

ledge of such variations is of obvious importance for the evaluation of various physiological and pharmacological experiments and a wide survey of the animal species was consequently judged necessary The demonstration of adrenergic fibres at some unexpected sites notably the cornea prompted an investigation of embryonic tissues to ascertain whether adrenergic fibres normally appear during embryonic life Special studies were further devoted to the relationships between adrenergic nerves and blood vessels as well as some other structures Most of the work presented in this review has been published in previous papers (Ehinger 1964 b 1966 a—f Ehinger and Falck 1966 Ehinger and Sporrong 1966)

MATERIALS AND METHODS

Eyes from the following adult animals were used about 120 mice about 240 rats 70 guinea pigs 8 golden hamsters 61 rabbits 5 sheep 9 dogs 17 cats 11 pigs and 25 cynomolgus monkeys Human eyes (17) were obtained by surgery necessitated for various reasons (see Ehinger 1966 b) Eyes in various stages of development were obtained from the embryos of 2 dogs 5 rats 5 guinea pigs and 11 humans

The fluorescence technique of Falck and Hillarp (see Falck and Owman 1965) was used for the histochemical localization of the "bio genic monoamines" dopamine adrenaline noradrenaline and 5 hydroxytryptamine The method is sensitive and precise and the under lying chemical reactions are well known (Falck Hillarp Thieme and Torp 1962 Corrodi and Hillarp 1963 and 1964) The specificity of the procedure when performed under proper precautions is excellent and may be checked in several ways with a number of tests and controls (cf Falck and Owman 1965) which were widely used in the present work

Holmstedt s (1957) modification of the Koelle technique was used for demonstrating cholinesterases The inhibitors used were isoOMPA Mipafox and NU 683 for the demonstration of acetylcholinesterase (AChE) in the various species and BW 284 C 51 for the demonstra tion of non specific cholinesterases (ChE) The difficulties in establish ing the specificity of the cholinesterase reaction have been discussed (Ehinger 1966 e) It was concluded that the specificity of the techni ques used was sufficient to obtain a resonably accurate demonstration of acetylcholinesterase which in nerves is thought to occur mainly in the cholinergic type The drawbacks of the technique (see Ehinger 1966 e) should however always be borne in mind and when possible the results should be evaluated in combination with supplementary studies

Elucidation of the relations between adrenergic and cholinergic fibres necessitated a technique permitting demonstration of adrenergic

and cholinesterase containing fibres in one and the same slide. Such a technique was obtained by a modification (Ehinger and Falck 1966) of the *fluorescence* technique. With this modified technique the cholinesterases remain sufficiently unaffected as to allow subsequent demonstration. The study also required selective parasympathetic denervations as well as a reliable technique for methylene blue staining for the positive identification of the parasympathetic and sympathetic fibres. Rats were subjected to parasympathetic denervation of the eye by a specially devised simple and rapid technique for ciliary ganglionectomy (Ehinger and Falck 1966). The selectivity of the procedure was apparent from the persistence of adrenergic nerve fibres, as demonstrated with the fluorescence technique. The technique for methylene blue staining of the nerve fibres of murine irides was standardized according to Hillarp (1946).

Intravascular injection of Indian ink (Ehinger 1966 d) made it possible to demonstrate the vessels simultaneously with the fluorescent fibres, giving a good view of their connections.

I NORMAL DISTRIBUTION OF ADRENERGIC NERVES

All the adrenergic fibres to the extra retinal ocular and orbital tissues originate in or pass through the homolateral superior cervical ganglion (Ehinger 1964 b 1966 a—c) All these fibres emitted a fluorescence characteristic of a primary catecholamine (see Falck and Owman 1965) which should thus be noradrenaline Most fibres seem to pass via the internal carotid plexus and to reach the eye via the trigeminal nerve since in the cat (3 animals) almost all fibres disappear when the connections between the carotid plexus and the semilunar ganglion or the postganglionic trigeminal branches are severed intracranially (Ehinger and Santini unpublished observations) Neither did any fluorescent nerve fibres appear when sympathectomized animals were given l dopa preceded by a monoamine oxidase inhibitor (nialamide) except in the ciliary ganglion (Ehinger 1966 b) and in the retina Such treatment induces very intense fluorescence in adrenergic fibres if present (Dahlström and Fuxe 1964 Owman 1964 b Hamberger Malmfors Norberg and Sachs 1964 Malmfors 1965 b Owman and Sjöstrand 1965 Falck and Owman 1966)

CORNEA

In all subprimate corneae hitherto studied varicose adrenergic fibres have been found in somewhat different numbers (Ehinger 1964 a and b 1966 a—c Laties and Jacobowitz 1964 Malmfors 1965 a) running either singly or especially in the periphery of the cornea in nerve trunks The density of the fibres is the same in central and peripheral regions In all adult animals except those with a comparatively thin cornea such as the rat and the mouse the adrenergic fibres occur only in the outer stromal layers Remarkably near full term embryonic corneae contain adrenergic fibres in a subepithelial plexus from which

fibres issue into the epithelium (Ehinger 1966 c). The appearance is highly suggestive of an epithelial innervation. The overall density of the fibres in the other parts of the cornea is also higher than in the adult animal. The fibres differed in appearance from those in the adult: the intervaricose segments fluoresce more intensely than in the adult and give the fibres a smoother appearance. The corneal fibres were never seen to accompany vessels. This has been confirmed in the experiments with intravascular injections in the adult (Ehinger 1966 d). Because of this and the regular occurrence of the corneal adrenergic fibres in all subprimate animals studied, and because they occur in the outer stromal layers only, and occur also in the embryo, it may be safely concluded that they are a normal constituent of the subprimate cornea, a matter of some controversy in the past (cf. Ehinger 1964 b). Adrenergic fibres have never been observed in normal adult primates, though it cannot be excluded that a few may occur concealed by the rather intense autofluorescence. Corneal adrenergic fibres occur in the human embryo (Ehinger 1966 d) and possibly a few of these can persist to adult age.

In the limbus of all species studied (Ehinger 1964 b, 1966 a—d), there is a network of adrenergic fibres, a part associated with the local vessels and a part forming a separate plexus between these vessels. The fibres occur both in the loose connective tissue of the conjunctiva and in the superficial layers of the limbal cornea. The number of fibres is smallest in primates.

The embryonic corneae offered excellent opportunities for studying the course of single nerve fibres over considerable distances (Ehinger 1966 d). These fibres were seen to branch repeatedly at their terminals, spreading over a large area. At the same time the density of the sub-epithelial network, when best developed, left no doubt that every basal epithelial cell was reached by several nerve fibres. Thus, also in the embryo the distribution of adrenergic fibres corroborated *Hillarp's* (1959) principle of convergence and multiple distribution of the terminal nerve fibres in the autonomic nervous system.

The observation that adrenergic fibres assume the terminal varicose appearance already while they are still running in the nerve trunk applies not only to the cornea (Ehinger 1966 d), but also to other parts of the eye (Ehinger 1964 b, 1966 a—c) and to other organs (Owman 1964 a, Falck *et al.* 1965, Owman and Sjöstrand 1965). Such varicosities do not differ morphologically from those occurring on the nerve fibre at e.g. smooth muscle cells.

This observation of varicose adrenergic fibres in nerve trunks does not confirm the postulate of Norberg and Hamberger (1964) that "the axons assume the typical appearance of varicose terminals precisely where they establish contact with the effector cells and enter the autonomic ground plexus" It is of course possible that a transmitter release occurs within the nerve trunks but it remains to be proved whether the amines stored in these varicosities in nerve trunks are immediately available for transmission

The biological significance of the corneal adrenergic fibres is obscure A regulating influence on the sensory nerves has been suggested (Laties and Jacobowitz 1964) The possibility of a more general metabolic influence has also been discussed (Ehinger 1966 e)

CHAMBER ANGLE

The outflow of the aqueous humour is generally thought to pass through the chamber angle giving obvious importance to the morphology of this region The density of the adrenergic fibres in the chamber angle of subprimates falls into three main classes

1 In the first group there are only few adrenergic fibres scattered in the loose tissues around the spaces of Fontana (Fig 1) Mice rats swine (Ehinger 1966 c) rabbits (Ehinger 1964 b) golden hamsters and sheep (Ehinger unpublished) belong to this group
2 In the second group most of the tissue strands of the chamber angle contain an adrenergic varicose fibre generally running radially (Fig 2) The fibres often though not always tend to be denser in the parts next to the sclera Cats and dogs belong to this group (Ehinger 1966 a and c)
3 The third type has so far been found only in the guinea pig (Ehinger 1964 b) Adrenergic varicose fibres occur abundantly in the loose connective tissue of the anterior part of the ciliary body Two or more adrenergic varicose fibres usually run together (Fig 3)

In primates no adrenergic fibres have been identified in the scleral trabeculae in the chamber angle despite a meticulous search in specimens where maximum fluorescence had been induced by the administration of l dopa preceded by a monoamine oxidase inhibitor (nialamide) and despite extensive investigations with the borohydride reduction technique of Corrodi Hillarp and Jonsson (Ehinger 1966 b and c)

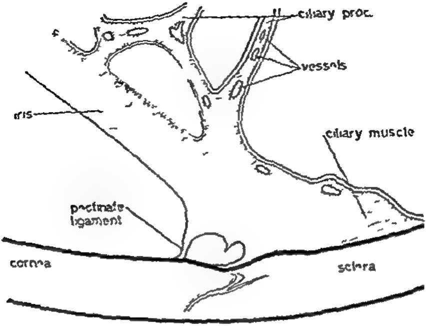

Fig 1 Schematic drawing of the chamber angle rabbit Adrenergic fibres dotted At the posterior surface of the iris the dots shown between the two lines represent the adrenergic fibres found within the dilator muscle For further explanations see the text.

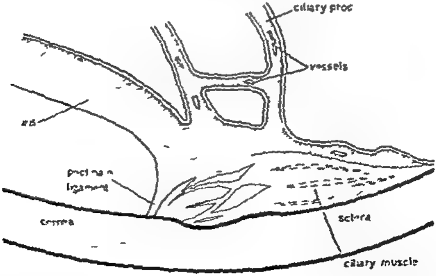

Fig 2 Schematic drawing of the chamber angle dog Adrenergic fibres dotted. At the posterior surface of the iris the dots shown between the two lines represent the adrenergic fibres found within the dilator muscle For further explanations see the text.

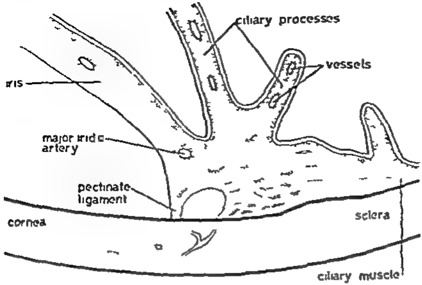

Fig 3 Schematic drawing of the chamber angle guinea pig For explanations, see the text

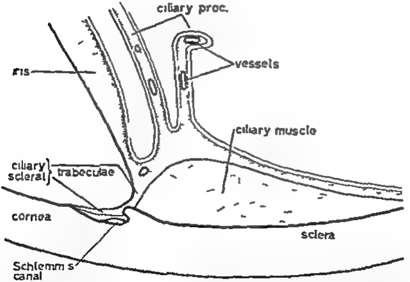

Fig 4 Schematic drawing of the chamber angle primates Adrenergic fibres dotted At the posterior surface of the iris the dots shown between the two lines represent the adrenergic fibres found within the dilator muscle For further explanations see the text.

However the intense autofluorescence of the scleral trabeculae makes it difficult to exclude completely the presence of adrenergic fibres in this region. The uveal part of the trabeculae, on the other hand, contains some meridionally and radially directed varicose fibres (Fig. 4). No adrenergic fibres have been found along the canal of Schlemm but some adrenergic varicose fibres occur further out along the aqueous drainage channels (Ehinger 1966 b).

There is unequivocal evidence that a mechanism sensitive to sympathomimetics can influence the resistance to aqueous outflow in the rabbit (for references see Bárány and Gassman 1965, Langham 1965, Paterson 1966). Pharmacologically the mechanism has been localized to the chamber angle (Bárány 1962, Sears and Sherk 1964). The paucity of adrenergic fibres in the tissues around the spaces of Fontana in the rabbit (Ehinger 1964) was therefore a rather unexpected finding, especially when contrasted with the rich supply in the guinea pig. Likewise, in experiments where maximum fluorescence had been induced in the adrenergic fibres by the administration of l-dopa preceded by nialamide, only few adrenergic fibres could be revealed in the chamber angle of rabbits, rats and mice (Ehinger 1966 c). If it be assumed that there are many adrenergic receptors in the filtering tissue of the chamber angle in rabbits, most of them can obviously not be in direct contact with adrenergic fibres. This situation is unusual but not unique: adrenergic fibres are lacking in umbilical arteries (Gennser, Owman and Sjöberg, unpublished observations), in the rat uterus (Owman and Sjöberg 1966), in the bronchial muscles (Larsson *et al.* 1966), and in some teleost hearts (von Mecklenburg, personal communication), but adrenergic receptors are well known in the rat uterus and bronchial muscles and have also been demonstrated in the umbilical arteries (see e.g. Eliasson and Åström 1955) and in the heart of a teleost (plaice, Falck *et al.* 1966).

The response in the mechanism which influences the resistance to aqueous flow is unusually sluggish. Little immediate effect was seen on the facility when the sympathomimetics were injected into the anterior chamber (Bárány, personal communication), whereas an increase in facility was observed after more slowly acting intravitreal injections (Eakins 1963, Eakins and Ryan 1964) and conjunctival injections (Sears and Sherk 1964, Gnädinger and Bárány 1964). Difficulties have also been encountered in classifying the receptors in terms of the well known α- or β-type (Gnädinger and Bárány 1964). There

is thus evidence other than the morphological that these receptors may have unusual characteristics

The sensitivity to intraocular injections of noradrenaline on the aqueous outflow facility has been shown to be increased after sympathectomy (Eakins and Ryan 1964 Sears and Sherk 1964) but this sensitizing effect of sympathetic denervation should not be accepted without hesitation as an evidence of a direct sympathetic innervation to the chamber angle Adrenergic nerves possess extremely effective uptake and storage mechanisms for amines as noradrenaline and the iridic adrenergic nerves have been shown to increase their transmitter content several times over when the substance is injected into the anterior chamber (Malmfors 1965 b) Very likely in normal animals the main part of the amines injected never reached the chamber angle but were immediately taken up into the adrenergic nerves when injected In the denervated eye on the other hand (and in the cocainized eye Eakins and Eakins 1964 Malmfors 1965 b cf also Paterson 1966) there is no such uptake mechanism and the main part of the injected amines reach the chamber angle resulting in readily measurable effects on the outflow facility This sequence of events presumably helps to explain the delay in the response to intravitreal injections of sympathomimetics (cf Eakins 1963 Eakins and Ryan 1964) 100 µg noradrenaline is certainly an overdose for the storage mechanisms in the intraocular adrenergic nerves but this overload will be apparent only some time after the injection when sufficient amounts of the drug have reached the anterior chamber

IRIS

Apart from the sphincter muscle and a possible distribution of the fibres to some special iridic melanophores in the cynomolgus monkey the adrenergic nerve fibres in the iris have principally the same arrangement in all species studied In the small animals with a very thin dilator such as rats mice guinea pigs and hamsters the adrenergic fibres form a dense lattice situated entirely outside and in front of the dilator but in the larger animals primates included they are found also within the muscle (Ehinger 1964 b 1966 a—c) Apart from the exceptions given below the abundance of adrenergic fibres in such that every muscle cell is surely reached by several adrenergic fibres Confirming descriptions of the normal morphology have been published (Laties and Jacobowitz 1964 Malmfors 1965 a) In animals with a slit

ments with intravascular injections (Ehinger 1966 d). In the ciliary processes the vessels and the epithelium lie so close to each other that it is difficult to differentiate between vascular and non-vascular fibres. However, tangential sections of processes show a plexus of adrenergic fibres extending beyond and between the vessels (Ehinger 1964 b, 1966 d). After inducing stromal oedema in the ciliary processes in rabbits to separate the vessels from the epithelium (Ehinger 1964 b), some fibres can be seen following the vessels, whereas others still lie close to the epithelium. Intravascular injection experiments in rabbits, rats and guinea pigs have shown the small vessels in the ciliary processes often described as capillaries to be accompanied and ensheathed by adrenergic fibres (Ehinger 1966 d) as distinct from most capillaries in other organs (Ehinger unpublished).

As judged from the pattern of the nerve distribution, an adrenergic innervation of the ciliary epithelium seems likely. In fact there is pharmacological evidence for such a possibility (Berggren 1965). Sympathomimetics have also been shown to influence the aqueous humour production in the intact eye (Weekers, Delmarcelle and Gustin 1965, Swegmark 1963, Eakins 1963, Langham 1965), but in these investigations a vascular reaction could possibly partake in the response.

The ciliary muscle varies widely from species to species regarding the number of adrenergic fibres (Ehinger 1964 b, 1966 a—c). In primates, pigs, sheep and guinea pigs there are some adrenergic fibres, although the meshes of the adrenergic network in primates (Ehinger 1966 b), pigs (Ehinger 1966 c) and sheep (Ehinger unpublished) are larger than in the other species and each of the meshes seems to enclose several muscle cells. Rabbits (Ehinger 1964) have fewer adrenergic fibres in the region of the ciliary muscle. In rats, mice (Ehinger 1966 c) and hamsters (Ehinger unpublished) the ciliary muscle is poorly defined and it was impossible to ascertain whether the scattered fibres found in the ciliary body have connections with any muscle cell. Only very few adrenergic fibres were seen in the main part of the ciliary muscle of the cat (Ehinger 1966 a) except a few posteriorly and near the sclera. A peculiar arrangement was found in the dog (Ehinger 1966 c), where most adrenergic fibres were situated in connective tissue strands interspersed chiefly in the anterior parts of the muscle. Only few varicose adrenergic fibres were seen in the smooth muscle bundles.

The influence of sympathetic nerves on the ciliary muscle has been discussed in the past, but in several species there is now cogent evi-

dence of adrenergic receptors in the ciliary muscle (see van Alphen et al. 1962, 1964 and 1965; Törnqvist 1966). In the monkey the muscle is innervated from the cervical sympathetic chain, and sympathetic stimulation causes a change in the accomodation towards hyperopia (Tornqvist 1966). Most likely the adrenergic fibres found in the present study in the ciliary muscle represent this innervation.

CHORIOID

In the chorioid a large part of the adrenergic fibres accompany blood vessels. Adrenergic fibres reach the choriocapillary layer and may even be seen between this layer and the pigment epithelium (Ehinger 1964 b, 1966 c; cf. also Malmfors 1965 a). Pharmacological and physiological evidence is consistent with a sympathetic innervation of the uveal vessels (cf. Bill 1962). Separate varicose adrenergic fibres may also be seen running in the stroma. In whole mounts of the rabbit and guinea pig chorioid it was evident that the fibre network was densest in areas corresponding to the visual streak respectively the central fundus (Ehinger 1964 b). Although a similar variation may be seen in the density of the vascular network, this variation seems not pronounced enough to explain the variations in the fibre network.

OPTIC NERVE

Apart from vascular fibres, the optic nerve is void of adrenergic fibres in the adult animal (Ehinger 1964 b, 1966 a—c; Malmfors 1965 a). Though less prominent, the adrenergic vascular plexus is of the same construction as elsewhere in the body. The adrenergic fibres cease rather abruptly where the vessels penetrate the cribriform plate.

RETINA

In the retina, adrenergic neurons have been described in the innermost part of the inner nuclear layer in rats and rabbits (Malmfors 1963; Haggendal and Malmfors 1963). In the rabbit retina, only dopamine could be detected in significant amounts (Haggendal and Malmfors 1965). The presence of the adrenergic neurons at the inner nuclear layer has subsequently been confirmed in all species investigated so far (Ehinger 1964 b, 1966 a—c; Malmfors 1965 a; Laties, personal communication). The fibres of this layer often form basket work forma-

tions around non-fluorescent perikarya in the innermost rows of cells of the inner nuclear layer. Occasionally, single varicose adrenergic fibres have been traced through the entire inner nuclear layer (Ehinger, unpublished observations). Furthermore, in the rabbit two additional adrenergic fibre layers were found (Häggendal and Malmfors 1965, Ehinger 1966 f). The different fibre layers in the rabbit may be called the *outer adrenergic fibre layer* (at the border between the inner nuclear and the inner plexiform layers), the *middle adrenergic fibre layer* (in the middle of the inner plexiform layer), and the *inner adrenergic fibre layer* (in the innermost part of the inner plexiform layer). A clearly defined middle adrenergic fibre layer was also found in the guinea pig, and a more scanty and variable middle adrenergic fibre layer in rats and in mice (Ehinger 1966 f). The adrenergic cells of the outer adrenergic fibre layer superficially resemble certain of the stratified amacrines of Cajal (1894). But unlike the fibres in the outer adrenergic layer none of the processes of the classical amacrines enclose any perikarya of the inner nuclear layer. Furthermore, the multitude of amacrines with different patterns of processes has no counterpart among the adrenergic cells, whose total number is also much smaller. Therefore, when discussing these cells it seems advisable for the time being to use the non-committal term *outer adrenergic cells*.

In addition to the initially reported outer adrenergic cells, adrenergic neurons were detected in the inner plexiform and the ganglion cell layers in all animals studied in the present investigation (Ehinger 1965, 1966 f and unpublished observations). The cells of the inner plexiform layer are the scantiest. They are more or less star-shaped and their processes not only ramify within the inner plexiform layer but also run to the other adrenergic fibre layers. They might be classified as interstitial amacrine cells (cf. e.g. Cajal 1894) but observations with phase contrast optics revealed that only part of the cells occurring in the inner plexiform layer are adrenergic. It was consequently considered necessary to distinguish the adrenergic cells in the inner plexiform layer from the interstitial amacrines, and the term *eremite cells* was therefore proposed (Ehinger 1966 f). Laties (personal comm.) has recently detected them also in the squirrel and the spider monkey.

The adrenergic cells of the ganglion cell layer always show a process extending to the outer adrenergic layer (Ehinger 1966 a, b, f). In primates the process is generally single and rather coarse; in other animals it may vary in thickness and in the degree of arborization. In a few cases some processes mingled with the ganglion cells. No further

contacts have been observed but it is well known that preterminal non varicose axons in both the central nervous system and in peripheral tissues often do not contain sufficient amounts of the transmitter to be readily visible. These cells have been called *alloganglionic cells* to distinguish them from other ganglion cells. It is not known whether they conduct centrifugally or centripetally. Synaptic end buttons occur only rarely on the cells in the ganglion cell layer and seem mainly to stem from intraretinal neurons (Polyak 1941). Centrifugal fibres are generally said to run to the inner plexiform or nuclear layers. If the alloganglionic cells conduct centrifugally they would consequently require unusually long dendrites all the way up to the brain. On the other hand it is widely believed that all ganglion cells degenerate after division of the optic nerve (cf. e.g. Kupfer 1963). If they do it would favour a centripetal direction of conduction. However the alloganglionic cells are so few that they may have been overlooked in the studies on the degeneration of the ganglion cells.

Cajal (1896) described a special type of amacrines (amacrines déplacées) occurring in the ganglion cell layer. They were found most abundantly in birds and reptiles and showed a narrow process extending to the middle of the inner plexiform layer where they ramified abundantly. The alloganglionic cells (Ehinger 1966 f) do not resemble these cells. Their process always reaches the outer adrenergic fibre layer and this process is generally much coarser than that shown by Cajal. Furthermore in pigeons and turtles the alloganglionic cells were not found to be more numerous than in mammals (Ehinger, to be published). There is thus no reason to identify the alloganglionic cells with Cajal's "amacrines deplacees" even if the alloganglionic cells should prove to be entirely intraretinal.

Along the retinal vessels only very few adrenergic fibres appear (Ehinger 1966 a—f). It should be noted that this is the only region in the eye where there are few adrenergic fibres along the arteries.

ORBITAL TISSUES

In the extraocular orbital tissues the smooth muscles have been shown to contain many adrenergic fibres. The orbital muscle has a rather widemeshed plexus often with a sheath of adrenergic fibres on the muscle surface (Ehinger 1966 c). The fibre density is higher and more uniform in the nictitating muscle (cf. Ehinger 1966 a). The lacrimal glands vary from species to species. Rabbits as well as rodents

such as mice rats and guinea pigs lack adrenergic fibres (except along vessels), whereas adrenergic varicose fibres can be found among the acini of the lacrimal glands of dogs cats pigs sheep and monkeys (Ehinger 1964 b 1966 a—c) The various glands of the eyelid do not contain any adrenergic nerve fibres except in connection with the smooth muscles they contain e g in the gland of Meibom in mice rats and rabbits (Ehinger 1964 b 1966 c)

CILIARY GANGLION

In the ciliary ganglion some neurons regularly fluoresce intensely after the administration of l dopa to animals pretreated with a mono amine oxidase inhibitor (nialamide) (Ehinger 1966 b) Thus these cells show certain similarities with adrenergic neurons elsewhere in the body (cf e g Norberg and Hamberger 1964 Malmfors 1965 b Falck and Owman 1966) However in most cases there are no fluorescent perikarya and only few varicose adrenergic fibres in the normal ciliary ganglion An exception to this rule was found in five young dogs all from the same litter in which all the ciliary ganglia abounded with fluorescent neurons and varicose fibres Repeated attempts to repro duce this observation have failed Hamberger Norberg and Ungerstedt (1965) have described varicose terminals occurring in the cat densely arranged around some non fluorescent perikarya of the normal ciliary ganglion This could not be confirmed in the present study (9 cats) in spite of the fact that the outcome of the fluorescence reaction was excellent in e g vascular fibres within the ganglion However in goats (2 animals Ehinger unpublished observations) varicose terminals have been found building dense basket work formations around some non fluorescent nerve cells The only conclusion to be drawn at present is that the classical concept of the ciliary ganglion as a purely choliner gic parasympathetic structure needs reconsidering

II NORMAL DISTRIBUTION OF CHOLINESTERASE CONTAINING NERVES

Albinotic rabbits rats and guinea pigs were studied (Ehinger 1966 e) Acetylcholinesterase containing fibres (AChE fibres) run radially in the corneal stroma in thick bundles giving off small branches to all parts of the stroma After prolonged incubation fine AChE fibres appear

within the corneal epithelium with connections to the stromal bundles of AChE fibres (Laties and Jacobowitz 1964, Peterson *et al.* 1965, Ehinger 1966 c). These intraepithelial fibres are presumably sensory (cf. Ehinger 1966 e). It has been shown by other methods that the perikarya and processes of sensory cells contain fairly equal and well demonstrable amounts of AChE (Giacobini 1959).

Only minor numbers of AChE fibres were found in the chamber angle in any of the species studied. In the guinea pig, the density of the nerve fibres seen when no inhibitors were used (to demonstrate also e.g. non specific cholinesterases, which occur also in adrenergic nerves) approached that seen in preparations studied for adrenergic fibres (cf. Ehinger 1964 b). This strongly suggests that the present technique is able to differentiate between AChE fibres and others. Further evidence of the specificity of the technique is discussed below (cf. also Ehinger 1966 c). The paucity of fibres in the chamber angle of rabbits differs somewhat from that described by Laties and Jacobowitz (1964), but in the present study fibre densities as described by them were found in the ciliary body, behind the tissues immediately surrounding the spaces of Fontana. These fibres are most probably related to smooth muscle cells (Ehinger 1966 c).

In the iris AChE fibres have been found abundantly in the sphincter muscle and, unexpectedly, also in the dilator, which is generally regarded as being innervated by sympathetic fibres only (cf. e.g. Adler 1965, p. 201). Additional evidence of its rich parasympathetic nerve supply will be discussed. No AChE fibres appear around the vessels, which is further evidence of the specificity of the method. In the rat the presence of a rich plexus of parasympathetic fibres to the dilator region was confirmed by parasympathetic denervation (extirpation of the ciliary ganglion) and subsequent AChE staining as well as methylene blue stainings of irides after chronic sympathetic denervation (Ehinger and Falck 1966). Moreover, in mice studies discussed in further detail below (Ehinger and Sporrong 1966) indicate the presence of cholinergic fibres as well as adrenergic in the dilator region of the iris. In cats also there is histochemical (cf. below) and pharmacological (Schaeppi and Koella 1964 b) evidence of a cholinergic innervation of the dilator.

From the layer of AChE fibres at the dilator such fibres extend into the iridic stroma. The similarity to the arrangement of the adrenergic fibres is striking (cf. Ehinger 1964 b and 1966 a—d). The structure innervated by these fibres is still unknown.

In the ciliary body, a plexus of AChE fibres similar in distribution to the adrenergic fibres may be seen under the epithelium although the AChE fibres are sparser (cf Ehinger 1964 b 1966 c) The AChE fibres are situated under the pars plana as well as in the ciliary processes The number of AChE fibres along vessels at the pars plana is low and in no way comparable to the number of adrenergic fibres superimposed on the media of the vessels (cf Ehinger 1966 c and d)

The ciliary muscle is clearly supplied with AChE fibres in rabbits, guinea pigs and rats (Ehinger 1966 e) this confirms the conception of its rich parasympathetic innervation The double supply of vegetative fibres in guinea pigs is noteworthy and most probably occurs also in pigs sheep rats mice rabbits and primates (Ehinger 1964 b 1966 a—d)

In the choroid the AChE fibres may be seen both in connection with arterioles and free in the stroma (Ehinger 1966 e) No AChE fibres were detected in the optic nerve

Pharmacologically cholinergic vasodilator fibres have been inferred at the "resistance vessels in skeletal muscle (cf e g Linden 1963) In the eye both methylene blue staining after chronic sympathetic denervation (rats Hillarp 1946 Ehinger and Falck 1966) and AChE stainings (rats guinea pigs and rabbits Ehinger 1966 e) have failed to show any such fibres along vessels of the anterior uvea However in the choroid AChE fibres that may represent such vasodilators occur along the arterioles (Ehinger 1966 e)

In the retina the presence of AChE containing layers (cf e g Esila 1963) has been confirmed (Ehinger 1966 e) In all species AChE occurs in the inner plexiform layer The position is similar to that occupied by the outer adrenergic fibre layer and cells (Ehinger 1966 f) and the amacrine cell bodies Unfortunately the resolving power of the cholinesterase technique did not permit more precise localization of the enzyme

In the orbital smooth muscles AChE fibres occur in all species studied (Ehinger 1966 e) Their number is smaller than that of the adrenergic fibres again proving the specificity of the technique The number is fairly equal in the three species studied but differences have been recorded between cats and rabbits (Jacobowitz and Koelle 1965)

The parasympathetic innervation of the lacrimal gland seems widely accepted (cf McEwen 1962 Rohen 1964) accordingly it also comprises AChE fibres in all species studied (Ehinger 1966 e) It may be concluded that rabbits as well as such rodents as mice rats and guinea

pigs have a single cholinergic innervation of this gland whereas others (pigs, cats, dogs, sheep and monkeys) have an adrenergic (Ehinger 1966 a—c and unpublished observations) as well as probably also a cholinergic innervation. The adrenergic fibres come from the cervical sympathetic chain (Ehinger 1964 b, 1966 a and c) and it may be supposed that the cholinergic fibres are mainly parasympathetic (cf. above).

III. STUDIES ON THE RELATIONS BETWEEN ADRENERGIC AND PARASYMPATHETIC NERVE FIBRES IN OCULAR TISSUES

Since the investigation required a large number of animals subjected to ciliary ganglionectomy, a fast and simple surgical procedure was devised for this operation on rats (Ehinger and Falck 1966). It was shown that after ciliary ganglionectomy only few AChE fibres remain in the dilator region of the murine iris, whereas the adrenergic fibres remain without observable change. This is in itself a proof for the existence of parasympathetic fibres in the dilator region (cf. above); it also clearly demonstrates that the adrenergic and AChE fibres are separate entities. Obviously, it is immaterial in this connection whether the enzyme demonstrated is really true AChE or a related enzyme. With a specially developed technique that permits the subsequent demonstration of adrenergic fibres and AChE fibres in one and the same slide, it was further shown that the adrenergic and cholinesterase containing networks are largely congruent (Ehinger and Falck 1965 and 1966, Eränkö and Räisänen 1965). It was concluded that the autonomic ground plexus of the rat iris is made up of separate adrenergic (sympathetic) and cholinesterase containing (mainly parasympathetic) fibres which, however, run concomitantly with surprising regularity (Ehinger and Falck 1966). Indeed, there are inconspicuous but definite differences between the adrenergic and AChE containing fibre networks corroborating that they are separate entities: in some places short segments of non-vascular adrenergic fibres could be seen without any accompanying cholinesterase activity, and somewhat more extensively also *vice versa*. Further proof of the duplication of the nerve supply was obtained in a study on the iris of mice (Ehinger and Sporrong 1966). It is known that in the ground plexus of this structure the adrenergic fibres usually run singly (Malmfors 1965 a, Ehinger 1966 c). Methylene blue staining, on the other hand

(Ehinger and Sporrong 1966) showed that usually two or more beaded nerve fibres run together. Except for possibly some few cholinergic fibres from the sympathetic chain, the non-adrenergic fibres should be parasympathetic (cholinergic) or sensory. As there is no reason to assume that sensory fibres run to this extent in the autonomous ground plexus, it may be concluded that adrenergic and cholinergic fibres run together also in the mouse iris. Recent studies in cats (Ehinger, unpublished observations) have also demonstrated a rich network of AChE fibres in the dilator muscle. These fibres degenerate after ciliary ganglionectomy, whereas the adrenergic fibres remain intact, proving that adrenergic and cholinergic nerves are separate entities also in the feline dilator.

Nothing in the experiments summarized above favours the idea of involvement of a cholinergic mechanism of the adrenergic fibres in their liberation of catecholamines (cf. Burn and Rand 1965). Indeed, it was shown that in the vascular adrenergic nervous plexus of the rat iris there is no AChE activity (Ehinger and Falck 1965 and 1966) and other regions have been found where there are many adrenergic fibres but where the AChE fibres are less densely arranged, e.g. in orbital smooth muscles (Jacobowitz and Koelle 1965, Ehinger 1966 c) and the vas deferens (Jacobowitz and Koelle 1965, Owman and Sjöstrand 1965 and unpublished) of certain species. Furthermore, a recent study on the choline acetylase activity in selectively denervated cat irides (Ehinger *et al.* 1966) showed that adrenergic fibres lack detectable amounts of this enzyme. The presence of choline acetylase may be regarded as the best available criterion of a cholinergic mechanism, which thus seems to be absent in most adrenergic fibres of the iris.

Pharmacological studies on several organs have produced evidence that adrenergic and cholinergic fibres of peripheral tissues are separate but may interact (Leaders 1963, 1965) and the findings discussed above present a morphological basis for such an assumption. It should be noted, however, that the two types of fibres do not invariably run together, as is evident from the fact that fibres of the one kind may be numerous in places where the other type is scanty (cf. above).

The experiments discussed above do not entirely exclude the possibility that a few fibres possess both a cholinergic and an adrenergic mechanism. It should be noted that some of the adrenergic ganglion cells of the superior cervical ganglion of the rat may contain AChE (Eränkö and Härkönen 1964).

IV. ADRENERGIC BARRIER MECHANISMS IN THE EYE

An enzymatic "blood brain barrier" has recently been demonstrated in the capillaries of the central nervous system. Dopa and 5-hydroxytryptamine are taken up by the endothelial cells, decarboxylated to the corresponding amines, and degraded by monoamine oxidase. However, it was shown that the amines themselves cannot pass through the luminal membrane of the brain vessels (Bertler *et al.* 1964 and 1966). In the murine eye the retinal capillaries have the same properties (Bertler *et al.* 1964 and 1966, Ehinger 1966 c), but not the uveal vessels (Ehinger 1966 c). Instead, the mentioned amino acids and the amines seem to pass more or less readily out into the aqueous humour.

Davson (1956) regarded the blood-aqueous and blood-liquor "barriers" as fundamentally similar and also postulated close diffusional connections between the extracellular brain tissue and the cerebrospinal liquor. From the present observations it is evident, however, that these deductions must not be extended to imply that there are no differences in the biochemical transport mechanisms in the retinal and uveal capillaries.

uously fewer than the adrenergic fibres. All lacrimal glands studied receive a rich supply of AChE fibres.

From selective denervations, combined demonstration of AChE and noradrenaline, and methylene blue staining on murine irides, it was concluded that the autonomous ground plexus of the dilator muscle is built up of concomitant adrenergic and cholinergic fibres. Only rarely do adrenergic and cholinergic fibres occur independently in the vegetative ground plexus of the dilator region. There was no evidence of a cholinergic mechanism in the majority of the adrenergic fibres.

In the retina the capillaries have the same capacity to act as a barrier for certain amines and their immediate precursors as in most other parts of the central nervous system. However, no such mechanism was evident in the uveal vessels, and the above mentioned substances seem to pass readily into the aqueous humour, thereby establishing a clear difference between the blood-retinal and blood-aqueous "barriers".

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Quantitative Aspects of Blood Flow and Oxygen Uptake in the Human Forearm during Rhythmic Exercise

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CHAPTER I

Introduction

During muscular exercise oxygen must be supplied by the blood to the working muscles in proportion to their requirements and the carbon dioxide and heat produced must be removed. To this end the human body responds with a set of adaptatory reactions: the ventilation and heart rate increase and the muscle capillaries dilate. The increased cardiac output is distributed so that an adequate amount of blood reaches the working muscle. Cardiac output and oxygen uptake are closely adjusted to the intensity of work. As the oxygen requirements approach and exceed the capacity of the cardiovascular system for oxygen supply, the metabolic processes shift towards an anaerobic breakdown of carbohydrate. Lactate is then formed from pyruvate and reduced diphosphopyridine nucleotide is oxidized, thus maintaining glycolysis and the liberation of energy. For recent reviews on the physiological phenomena associated with muscular exercise in man, especially the physiology of the heart and the lungs, see Asmussen (1965) and Carlsten and Grimby (1966).

Local muscle circulation and oxygen uptake have been extensively investigated but, from necessity, often with indirect methods. Only in animal studies have direct observations been possible. The remarkable investigation by Chauveau and Kaufmann (1887) on the muscle that elevates the upper lip of the horse was one of the first to demonstrate increased blood flow and oxygen uptake during muscular activity. Kramer, Obal and Quensel (1939) used rhythmical stimulation of the gastrocnemius muscle of dog and recorded the venous oxygen saturation and blood flow with an optical method. During steady state exercise the blood flow and oxygen uptake were found to be linearly related to the work done. The rise in oxygen consumption was associated with an increased blood flow and unchanged venous oxygen saturation. Barger *et al.* (1956) confirmed these findings on the basis of indirect evidence from experiments on dogs running on treadmills. Kramer, Quensel and Schafer (1939) have demonstrated that even light work is accompanied by the production of small amounts of lactate. During heavy exercise the lactate production increased strongly. An extensive review on the circulation in skeletal muscle has recently been presented by Barcroft (1963).

In the animal experiments the uptake and production of metabolites per unit time were calculated from Fick's equation as the product of blood flow

arm blood flow, oxygen uptake and lactate production during exercise The dye dilution technique was chosen for measuring blood flow since it appeared for theoretical and practical reasons to be the method best suited for this type of study Before quantitative calculations could be performed it was necessary to further establish the origin of forearm deep venous blood during exercise

In brief, the aims of the present study were

to apply a method for the determination of brachial artery blood flow during rhythmic exercise,

to further establish the origin of deep venous blood of the forearm during exercise, and

to determine the forearm
during various types of exerc

CHAPTER II

General Procedures and Methods

Procedure

Catheterization. After the hand and distal forearm had been immersed in hot water for 5—10 min the radial artery was punctured at the level of the radial styloid process. A teflon catheter was introduced percutaneously (Seldinger 1953) 2—3 cm upstream. The brachial artery was punctured below or at the intercondylar line and a catheter inserted 2—3 cm in the proximal direction.

Teflon catheters were also introduced percutaneously into a deep and a superficial forearm vein. The deep venous system could usually be reached via the median cubital vein but in some cases the catheter was advanced through the cephalic vein. The catheter was manipulated during venous stasis until it had advanced so far upstream that its tip could not be palpated. The catheter had then been introduced approximately 5—8 cm. A superficial forearm vein, usually on the radial side, was punctured and a catheter inserted so far that its tip could be palpated approximately 5 cm below the intercondylar line.

Before catheterization the skin and subcutaneous tissue was infiltrated with ca. 0.5 ml 1 *per cent mepivacaine* solution (Carbocain®). Catheters inserted into arteries had an internal diameter in the tip and an external diameter of 0.5 and 0.9 mm respectively, the corresponding dimensions of the venous catheters were 0.9 and 1.2 mm. The tips of the catheters were adapted during heating to the dimensions of guides of known diameter. Care was taken to achieve a close fit. The diameters of the guides were checked with a micrometer.

Exercise. Rhythmic forearm exercise was performed in the supine position with the forearm horizontal (unless otherwise stated) at an angle of approximately 45° to the body. The elbow was extended and the volar side of the hand and the forearm faced upwards. The resting position of the hand ergometer handles was adjusted until the middle phalanges of the second, third and fourth fingers were approximately vertical.

The exercise was performed in time with a variable electric metronome. Three hand ergometers were used. One, employed in most of the experiments presented in Chapters III and IV, has been described by Pernow and Wahren (1962).

The position of the other beam on the oscilloscope screen was adjusted to correspond to the desired peak tension of contraction and the subject watched the oscilloscope continuously during exercise

Maximal force of finger flexion was tested according to Tornvall (1963) using a strain gauge dynamometer and a Philips PT 1200/01 measuring bridge The instruments were calibrated against known weights

Plethysmography A venous occlusion plethysmograph was used for the determination of total forearm and hand blood flow The hand and forearm were encased in a double walled plexiglass tube closed at one end and sealed by a rubber cuff to the forearm at the other The seal to the skin was made at the level of the intercondylar line Water at a thermostatically controlled temperature (34° C Barcroft and Edholm 1946) was circulated in the space between the two plexiglass walls and the temperature inside the inner wall could be measured via a thermocouple connected to a mirror galvanometer (TE 3 Ellab) A small positive pressure (20—30 mm H_2O) was introduced into the air filled space around the hand and the system was then tested for leaks The pressure increase following venous occlusion was measured with a pressure transducer and registered on an ink jet recorder (EMT 33 and Mingograf 42 Elema Schonander) The system was found to be linear for pressure variations within the volume range used The coefficient of variation for a single determination of blood flow was 12 9 per cent This value was calculated from duplicate determinations within 30—45 min in 10 subjects

Dye dilution curves Injection of 0 25—0 5 mg indocyanine green (Cardio green Hynson Westcott and Dunning) into the brachial artery was performed with a motor driven tuberculine syringe modified according to Sparling (1961) and Grimby and Nilsson (1963) The forward part of the syringe extended into a trunk After all but 0 1 ml of the syringe had been filled with saline dye (0 25—0 5 mg in 0 1 ml) was drawn into the anterior part of its trunk and the tip was connected to the brachial artery catheter Thus on injection the dye was flushed into the artery with the saline following behind The kinetic energy per unit time during the injection was calculated to be 31 800 dynem/sec The linear velocity in the catheter tip was 320 cm/sec

Two cuvette densitometers were used (model X250 and X300 Waters Corp) connected to a Speedomax Type G recorder (Leeds and Northrup) The time for full scale deflection of the recorder was 0 3 sec Blood was drawn through the densitometer cuvette at a rate of 12—16 ml/min Calibration was performed for each subject using blood with at least two different dye concentrations

The *total passage time* after a single injection was determined from the recorded dye dilution curve as the time from injection to return of the curve

to the preinjection level Total passage time corresponds to the sum of appearance time and passage time as defined by Wood and Swan (1954) No correction was made for recirculating dye The curve was then cut out and balanced vertically with a pin on the baseline until the abscissa was horizontal The position of the pin on the baseline indicated the x co-ordinate of the center of gravity which was taken to be the *mean transit time* The mean transit time was corrected for the mean time of the collecting system (ca 2 sec) The frequency function of transit times through the collecting system was of short duration as compared to that of the vascular system and no correction was made when calculating the forearm total passage times

Venous pressure was measured with a pressure transducer (EMT 490A Elema Schönander) connected to an ink jet recorder The reference used for zero pressure was the mid thoracic point at the level of insertion of the fourth rib

Skin and blood temperatures were measured with a thermocouple and a mirror galvanometer (TE 3 Ellab) In skin temperature measurements the thermocouple was applied to the skin for ca 5 sec Recordings of blood temperature in the radial artery were made with a thermocouple mounted in the tip of a teflon catheter The thermocouples were calibrated against a precision mercury thermometer

Heart rate was determined from an electrocardiogram

[illegible] *fold thickness* was measured with a skinfold caliper (pressure 10 g/mm² [illegible]

[illegible] *of the forearm and the hand* were measured by water displace[illegible] circumference of the arm with the elbow extended was measured [illegible] at the thickest part of the forearm and just proximal to the radial [illegible] process The length of the forearm was taken to be the distance between [illegible] olecranon [illegible] d the radial styloid process

Statistical m[illegible]d Most of the statistical calculations were performed according to Snedecor (1956 The analysis of variance in Chapter III was computed as described by Dixon and Massey (1957) using a statistical model with two variables of classification and repeated measurements The regression lines were compared according to Hald (1962) Multiple regression analysis was performed on a Control Data Corporation 3600 computer with a BMD 02R stepwise regression program (Dixon 1964) The following probability (p) levels of significance were used $p < 0.001$*** highly significant $p < 0.01$** significant and $p < 0.05$* probably significant Unless otherwise stated the results given in the text are means ± one standard deviation (M ± SD

TABLE 1 The error for a single determination in various blood analyses expressed as the standard deviation (SD) for a single determination and the corresponding coefficient of variation The calculations are based on duplicate blood samples obtained consecutively and thus include errors of sampling and analysis as well as any spontaneous variation

	Range	SD	Coefficient of variation	n
Oxygen sat per cent	0—33	2.0	8.7	20
Oxygen sat per cent	34—87	3.3	5.4	10
Oxygen sat per cent	> 90	0.8	0.8	15
Haemoglobin g/100 ml	12.0—15.0	0.19	1.5	20
pO mm Hg	15—30	0.4	2.3	10
Haematocrit per cent	35—45	0.25	0.6	30
Lactate mmoles/l	0—1.0	0.08	10.8	30
Lactate mmoles/l	1.0—4.0	0.26	6.6	30
Indocyanine green mg/l	3.0—6.0	0.17	3.3	40
Indocyanine green mg/l	*6.0—8.5*	*0.29*	*3.5*	*40*

Blood analyses

Sampling Blood samples for the analysis of oxygen saturation haemoglobin concentration (4.5—5 ml) and viscosity (10 ml) were drawn into 10 ml silicon ized glass syringes The dead space in each syringe was filled with heparin solution (5 000 I U /ml Vitrum) The haemoglobin concentration values were corrected for dilution by the heparin All other blood samples were collected in disposable plastic syringes For error of methods see Table 1

Oxygen saturation was determined spectrophotometrically by a slightly modified form of the method described by Drabkin (1950) It was found that the presence of indocyanine green in blood (10 mg/l) did not influence oxygen saturation ($p > 0.3$) or haemoglobin concentration ($p > 0.6$) values

pO was measured with a macro-oxygen electrode (Beckman Spinco Division) reading directly on a Beckman model 160 physiological gas analyzer The electrode was mounted in a cuvette described by Holmgren (1965)

Haemoglobin concentration was analyzed by the cyanmethaemoglobin technique (Drabkin and Austin 1935) using a reagent and standard from Hycel Inc

Haematocrit was determined and corrected for trapped plasma (1 3 per cent) according to Garby and Vuille (1961) using a high speed micro-capillary haematocrit centrifuge (10 min 12,500 rpm) (Wifug Automatic 102—11)

Erythrocyte sedimentation rate (ESR) was determined according to Westergren (1924) at room temperature and read after one hour The reading was

corrected to a standard hæmatocrit (43 per cent) using Gram's chart (1928) *Viscosity* of blood and plasma was measured with a cone plate micro viscometer (Brookfield LVT) at 37° C, using the procedure described by Rand *et al* (1964) The viscometer was calibrated with silicone oil (viscosity 7 7 centipoise) The coefficients of variation for a single determination of blood viscosity at shear rates of 23, 46, 115 and 250 sec $^{-1}$ are reported to range from 2 2 to 3 3 per cent for the equipment used in the present study (Groth 1966) *Lactate concentration* was analyzed with an enzymatic method based on the techniques described by Hohorst (1962) and Lundholm Mohme Lundholm and Vamos (1963) Two ml blood was precipitated, within 30 sec of sampling with 2 ml ice chilled 0 6 M perchloric acid The samples were centrifuged for 5 min at 4,500 rpm The supernatant was then removed and centrifuged again for 5 min Perchlorate ions were removed by the addition of 0 3 ml 0 4 M K_3PO_4 to 1 5 ml of the supernatant After 10 min in an ice bath the precipitate was centrifuged down for 5 min at 4,500 rpm The supernatant extract of the blood was transferred into a stoppered glass tube Analysis of lactate was performed within 4—6 days

The extract (0 2 ml) was pipetted into a spectrophotometer cuvette together with 0 2 ml NAD (Boehringer) (0 02 g NAD/ml) and 2 ml buffer solution The buffer contained 5 2 g hydrazine sulphate, 7 5 g glycine, 0 2 g EDTA and 51 ml 2N NaOH per 100 ml solution The absorbance was read at 340 mμ against a blank of 2 2 ml buffer and 0 2 ml NAD in a Beckman DB spectrophotometer with a scale expansion unit and a recorder After the addition of 0 05 ml LDH (Boehringer, 2 5 mg enzyme protein in 1 ml 2 2 N ammonium sulfate) the cuvette was incubated at 25 $\pm$ 1° C for 40 min and the increase in absorbance was then determined

Blood lactate concentrations were calculated using a molar extinction co efficient for NADH at 25° C of 6 22 cm^2/μM (Horecker and Kornberg 1948) Lactate solutions of known concentrations were analyzed at regular intervals to check the chemicals used

Indocyanine green was analyzed at the point of maximal absorption (close to 805 mμ) using a Beckman B spectrophotometer The readings were made in serum obtained after clotting and centrifugation of 2 ml blood samples Clot retraction was promoted by incubating the blood samples for 15 min in a waterbath at a temperature of 37° C Severely hæmolyzed samples were discarded A calibration curve was made for each subject with readings at 4 and 8 mg/l

CHAPTER III

A Dye Dilution Method for the Determination of Brachial Artery Blood Flow during Rhythmic Exercise[1]

Subjects

The subjects were 38 healthy male volunteers (age range 20—42 years M = 25 2 years) Of these, eleven were firemen, four policemen and the remainder students

Procedure

The procedures for catheterization exercise plethysmography recording of dye dilution curves and blood analyses are described under General Procedures and Methods

Constant infusion

Indocyanine green in 6 per cent (unless otherwise stated) dextran solution (Macrodex® mean molecular weight ca 75 000) containing 0 9 per cent sodium chloride was infused in varying concentrations at rates of 0 6—1 5 mg dye/min A constant infusion pump was used with stepwise variable infusion rates and calibrated 100 ml glass syringes The diameter of the syringes differed slightly causing the infusion rates to vary within 3 6 per cent of the mean The syringes with dye solution were heated in a thermostatically controlled water bath set at 38° C for at least 10 min before the start of infusion

Experimental

Mixing of dye and blood

The rate at which the dye solution was infused into the brachial artery during exercise was varied stepwise between 4 5 and 52 ml/min After 2 min infusion 2 ml blood samples were drawn simultaneously from the radial artery a deep and a superficial forearm vein Blood sampling was done twice in rapid succession and was usually completed within 15—20 sec A total of 97 infusion periods were performed in 21 different subjects

The mixing of dye and blood was evaluated from the dye concentrations in the blood samples from the different sampling sites Agreement between the dye concentrations of the different samples indicates good mixing of dye and blood before the bifurcation of the brachial artery Discrepancies on the other

[1] A preliminary account of these results has been published earlier (Wahren 1965)

TABLE 2 Summary of data relating to mixing of dye and blood in the brachial artery at various infusion rates during exercise Dye concentrations were determined in duplicate blood samples obtained simultaneously from the radial artery a deep and a superficial vein The coefficient of variation for a single determination at each infusion rate was calculated from differences between two consecutive blood samples from the same sampling site F is the ratio between the total intraindividual variance for n sets of 6 dye concentration values obtained for each infusion period and the variance for a single determination n denotes the number of subjects The probability p that the total variance is the same as the variance for a single observation is indicated ($p < 0.01$** $p < 0.001$***) A high F value thus indicates non uniform mixing of dye and blood

Infusion rate (ml/min)	Kinetic energy/sec of infusion (dynem/sec)	Linear velocity of infusion (cm/sec)	F	n	Coefficient of variation for a single determination (per cent)
4.5	55	38	21.60***	4	5.5
10	610	85	10.37***	5	3.8
15	2030	125	1.82	7	10.6
23	7300	195	3.53**	8	4.0
34	23700	290	1.04	17	3.1
57	84600	442	0.81	7	3.4

hand suggest inadequate mixing in the brachial artery or the presence of an anomalous arterial supply

The six results for dye concentration during each infusion period were subjected to analysis of variance Results obtained in one individual were considered only once for each infusion rate, and only the set of values obtained at the lowest work intensity and hence the lowest blood flow was used in the statistical processing reported in Table 2 Fig 2 gives the results from all the experiments

It is apparent from Table 2 and Fig 2 that the mixing of dye and blood in the brachial artery during exercise varies with the rate of infusion An infusion of 34 ml/min was the lowest of those tested that gave satisfactory mixing i e no significant difference in the dye concentrations of blood samples from the radial artery a deep and a superficial vein Satisfactory mixing conditions were also indicated ($F = 1.07$) by the 5 — out of a total of 17 — sets of values for this infusion rate which represented the highest dye concentration values and thus the lowest blood flows

The values at 15 ml/min call for special comment The F values in Table 2 were computed using the variance for a single observation calculated separate

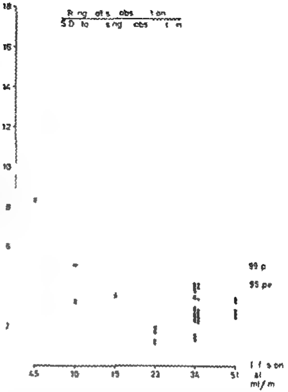

Fig ? The dye distribution in the forearm vascular system at varying infusion rates during exercise expressed as the ratio between the range for six dye concentrations (measured in duplicate blood samples from the radial artery a deep and a superficial vein) and the standard deviation for a single determination of dye measured at an infusion rate of 34 ml/min The 95 and 99 per cent confidence intervals are indicated

ly for each infusion rate This variance was unusually high at 15 ml/min Recalculation of the F value this time using the standard deviation for a single observation for the infusion rate 34 ml/min indicated a markedly uneven dye distribution (F = 6 9***) The high coefficient of variation for a single dye determination at 15 ml/min may have been caused by lability of the mixing conditions in the brachial artery especially since improved mixing conditions were found at an infusion rate of 23 ml/min

No significant difference was found between the dye concentrations of the first and the second blood sample from any of the sampling sites

Circulation times in the forearm

In dye dilution studies of this type the minimum length of the infusion period is determined by the circulation times from the brachial artery to the radial artery a deep and a superficial vein The measurement of representative indicator dilution concentrations requires that the infusion continues for longer than the longest total passage time for the sampling sites used Therefore total passage time and mean transit time were measured from the brachial artery to the radial artery a deep and a superficial vein during exercise

TABLE 3 Total passage time in sec for dye injected into the brachial artery and sampled in the radial artery, a deep vein and a superficial vein during light (3.6—6.0 kpm/min) and heavy (9.0—15.0 kpm/min) exercise with the mean (M), standard deviation (SD) and number of observations (n). p indicates the probability that the differences between the total passage times during light and heavy exercise are caused by random factors

	Radial artery		Deep vein		Superficial vein	
	Light	Heavy	Light	Heavy	Light	Heavy
M ± SD	13.7 ± 2.6	12.5 ± 1.5	43.8 ± 8.5	22.7 ± 3.0	70.6 ± 20.4	47.0 ± 14.4
n	10	6	12	10	11	7
p	> 0.2		< 0.001***		< 0.001***	

Single injection Dye dilution curves were recorded after the injection of dye into the brachial artery during light exercise (3.6—6.0 kpm/min) in twelve subjects and heavier exercise (9.0—15.0 kpm/min) in ten subjects. The curves were registered from one sampling site at a time during steady state exercise. The results are given in Table 3.

During light exercise the average mean transit time from the brachial artery to the radial artery was 4.4 sec, to a deep vein 15.7 sec and to a superficial vein 33.5 sec (n = 7). At heavier exercise the corresponding values were radial artery 3.4 sec, a deep vein 9.1 sec and a superficial vein 18.9 sec (n = 6).

Constant infusion The time from the start of infusion until equilibrium concentration is obtained is an estimate of the total passage time. During exercise (3.6—6.0 kpm/min) blood was sampled in five subjects from the radial artery, a deep and a superficial vein at 20—30 sec intervals from the start of dye infusion into the brachial artery. The infusion (34 ml/min) lasted for 2.5—3 min. The concentrations found were corrected for recirculating dye (see below) and the build up curves were plotted (Fig. 3). Equilibrium concentrations were reached rapidly in the radial artery, a little later in the deep vein and after a further interval in the superficial vein.

There was good agreement between the values for total passage time as determined from single injection and constant infusion techniques (Fig. 3). The results show that if all three sampling sites are used, the infusion should last [illegible] min at least during light exercise. If, however, samples are drawn only from the radial artery and a deep vein, 1 min will be sufficient at light exercise and give a margin at heavier work.

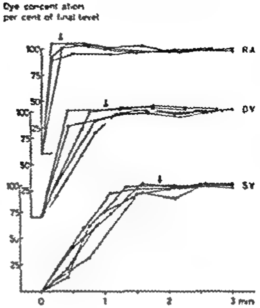

Fig 3 Build up curves for the dye concentration in blood from the radial artery (RA) a deep (DV) and a superficial vein (SV) from the start of infusion at 3½ ml min into the brachial artery during light exercise (3 6—6 0 kpm/min) Arrows indicate the mean values for total passage time estimated from dye dilution curves recorded after a single injection of dye

Recirculation

The concentration of recirculating indicator during infusion of dye was determined in frequent blood samples from the contralateral brachial artery in six subjects during exercise The duration of infusion was varied between 1 and 3 min and both light and heavy exercise were used (3 6—14 0 kpm/min)

The dye started to appear in a detectable concentration in the contralateral brachial artery 20—40 sec after the start of infusion The concentration then rose approximately linearly during the infusion and for 15—40 sec after this had been terminated (Fig 4 and 5) Once the peak had been reached the concentration fell off rapidly Five infusion periods of 1 min 15 sec each at intervals of 2 min 45 sec gave a final peak concentration that was 1 3—1 7 (n = 5) times higher than the first peak (Fig 5) The dye concentrations in the pair of blood samples obtained simultaneously from the radial artery and the contralateral brachial artery showed good agreement both when the samples were taken immediately before the start and 15—20 sec after the end of the infusion (Fig 5) The rising concentration of recirculating dye could be fairly accurately interpolated from the dye concentration readings in the radial artery blood samples

It is mainly the length of the infusion period which determines the correction for recirculating dye since the background concentration increases linearly during infusion However accurate correction for recirculation requires

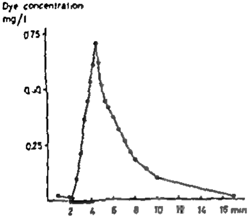

Fig 4 Recirculation during and after infusion of dye Dye concentration (mg/l) determined in blood samples from the contralateral brachial artery during light exercise and infusion (2 min 15 sec) of 0 9 mg/min of dye in one subject

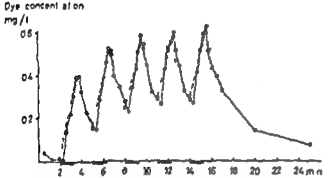

Fig 5 Recirculation during repeated infusions Dye concentration (mg/l) in blood samples from the contralateral brachial artery (●——●) during repeated infusion (1 min 15 sec) and the ipsilateral radial artery (○– – –○) before and 15—20 sec after the infusion period There is good agreement between the contralateral brachial artery concentration of recirculating dye during infusion and the background level interpolated from the ipsilateral dye concentrations in radial artery blood samples obtained immediately before and after the infusion period

that an amount be subtracted from the total dye concentration corresponding to the arterial background at one mean transit time before the sample was obtained (Zierler 1961) The mean transit time is dependent on the location of the sampling site and probably also on the magnitude of the blood flow Since it is unfeasible in practice to determine the mean transit time at each blood flow measurement standardized mean transit times were used for each sampling site namely the average of the mean transit time values recorded at high and low work intensity Consequently in the present study when blood samples were drawn during infusion of dye from the radial artery and a deep forearm vein the concentration of recirculating dye was interpolated to a point 10 sec before the time of sampling (see *Circulation times in the forearm single injection*) This approximation seems justified in that in the experiments on the mixing of dye and blood (see above) no significant difference

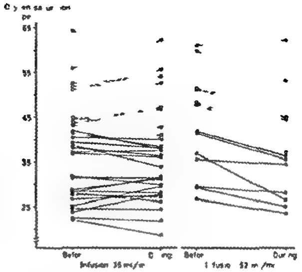

Fig 6 Effect of infusion on venous oxygen saturation Oxygen saturation in deep (●——●) and superficial (○– – –○) venous blood before and during infusions of 34 and 52 ml/min during light exercise

was found in the dye concentration in blood from the different sampling sites although no correction was made for recirculating dye

The interpolated background concentration constitutes only a relatively small part of the total dye concentration in forearm blood during infusion In the blood flow determinations reported in the section on reproducibility (see below Fig 11) the background amounted to 11 2 ± 3 7 per cent of the total concentration

Effect of infusion on blood flow

In dye infusion experiments of this type the infusion (F_I ml/min) itself forms a significant fraction of the total flow through the brachial artery during infusion (F_T) It is thus essential to know to what extent the infusion of dye interferes with the pre infusion brachial artery blood flow (F_B)

Several possibilities are at hand The addition of F_I to F_B may lead to a partial replacement of F_B by F_I i e $F_T < F_B + F_I$ (A) Rapid intraarterial injections have been found to elicit vasodilatation possibly owing to haemolysis This could cause $F_T > F_B + F_I$ (B) Lastly F_B may be unchanged during infusion $F_T = F_B + F_I$ (C)

The effect of the infusion on blood flow during exercise was analyzed from changes in oxygen saturation and haemoglobin concentration during infusion of dye using a 5 per cent dextran solution If $F_T = F_B + F_I$ then the venous oxygen saturation will be unchanged assuming a constant oxygen consumption and arterial oxygen saturation before and during the infusion period and a negligible addition of oxygen from the infused solution $F_T > F_B + F_I$ should

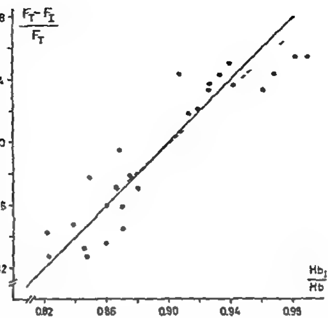

Fig 7 Effect of infusion on haemoglobin concentration Comparison between the ratios $F_T - F_I/F_T$ and Hb_I/Hb where F_T is the total flow through the brachial artery during exercise and an infusion of F_I ml/min (34 ml/min) and Hb and Hb_I are the haemoglobin concentrations before and during infusion. The regression line (broken line $y = 0.868x + -0.117$ SD = 0.019 r = 0.90***) does not deviate significantly from the line of identity ($p > 0.6$)

lead to a compensatory increase in venous saturation while the opposite is true for $F_T < F_B + F_I$

If again $F_T = F_B + F_I$ then the initial haemoglobin concentration (Hb) will be reduced during infusion to a level (Hb_I) proportional to the dilution of blood by the dye solution $Hb_I/Hb = F_B/F_T$ F_B is not known but may be substituted by $F_T - F_I$ The ratios can then be used to test the alternative $F_T = F_B + F_I$ For alternative (C) above the ratios would be identical by definition while (B) is indicated by $Hb_I/Hb > (F_T - F_I)/F_T$ and (A) by $Hb_I/Hb < (F_T - F_I)/F_T$

The oxygen saturation of blood samples from deep and superficial veins did not change significantly ($n = 21$ $n = 10$ $p > 0.3$) during exercise (4.0—15.0 kpm/min at an infusion rate of 34 ml/min (Fig 6) The same result ($n = 10$ $p > 0.4$) was obtained for values measured in deep venous blood during light exercise only (4.0—6.0 kpm/min) When on the other hand, an infusion rate of 52 ml/min was used during light exercise (4.0—6.0 kpm/min) there was a significant reduction of deep venous oxygen saturation (mean 4.6 per cent) (Fig 6) Superficial venous blood showed a probably significant

TABLE 4 Summary of some data (M ± SD) relating to the flow properties of blood before and during infusion of indocyanine green in 5 per cent dextran solution The blood and plasma viscosity (cP) at different shear rates (sec^{-1}) haematocrit (HCT per cent) and erythrocyte sedimentation rate (ESR mm) were all measured in blood samples from the radial artery before and during infusion The ESR was corrected for changes in HCT (Gram 1978) The blood samples were obtained during exercise at 5 and 10 kpm/min Differences were calculated from paired observations before and during infusion and were all highly significantly different from zero

	Viscosity (cP) at a shear rate (sec^{-1}) of				HCT	ESR
	23	46	115	230	per cent	mm
Blood n = 14						
Before	5.99±0.54	5.25±0.42	4.38±0.23	3.91±0.19	40.7±0.5	5.8±4.3
During	5.45±0.46	4.88±0.36	4.29±0.33	3.83±0.25	36.4±0.4	14.9±7.6
Change	−0.54±0.24	−0.37±0.15	−0.16±0.12	−0.13±0.09	−4.3±0.9	−9.1±4.8
Plasma n = 14						
Before	1.65±0.19	1.56±0.17	1.48±0.12	1.47±0.12		
During	1.84±0.26	1.76±0.16	1.61±0.13	1.64±0.11		
Change	+0.19±0.17	+0.20±0.13	+0.17±0.13	+0.17±0.12		

decrease Taken together the reduction in deep and superficial venous oxygen saturation was highly significant

The haemoglobin ratio Hb_i/Hb was measured in radial artery blood samples before and during infusion of 34 ml/min in six subjects at light exercise (4.0—6.0 kpm/min) Values were obtained during three separate infusion periods for each subject In a further fifteen subjects deep venous blood samples were also obtained before and during infusion of dye during a period of heavy exercise (9—15 kpm/min) F_T was calculated from equations (1) and (2) below The results are given in Fig 7 No significant systematic difference could be found between the two ratios (n = 33 $p > 0.2$) nor did the regression of $(F_T - F_I)/F_T$ on Hb_i/Hb deviate significantly from the line of identity No difference was found between the results for radial artery and deep venous blood

Effect of infusion on the flow properties of blood

Blood and plasma viscosity haematocrit and ESR were measured in samples from the radial artery before and during the infusion of dye in a 5 per cent dextran solution Fourteen infusions were performed altogether in seven sub-

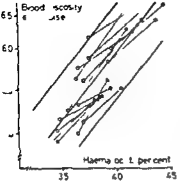

Fig 8 Effect of infusion (34 ml/min) on blood viscosity at a shear rate of 23 sec The regression $\pm$ 2 SD of log viscosity on haematocrit is shown ($^{c}\log y = 0.0294x + 0.6049$ SD 0 053 $r = 0.85^{***}$) The thin lines join measurements in blood samples obtained before (●) and during infusion (○) in the same individual

jects Whole blood viscosity always decreased ($p < 0.001$) during infusion, while plasma viscosity increased ($p < 0.001$) (Table 4) The changes were most pronounced at low shear rates The regression of log viscosity at shear rate 23 sec^{-1} on haematocrit is shown in Fig 8 A small but significant increase in ESR corrected for haematocrit variation was found in the samples obtained during infusion (Table 4)

Dye carrier solution

In infusion experiments of this type the dye should be dissolved in a colloid solution of such concentration that, when mixed with blood the shift in blood osmotic pressure is minimal Any osmotic effect is likely to be greatest at low flow rates

Dextran solutions of varying concentrations were infused for 1 min 15 sec into the brachial artery during light exercise (4 0 kpm/min) The haemoglobin concentration was measured in radial artery and deep venous blood immediately before the start and at the end of the infusion period The change

TABLE 5 Arterio venous haemoglobin concentration difference (Hb_{a-dv} g per 100 ml) measured during exercise in blood samples from the radial artery and a deep vein before and during infusion of 6 and 5 per cent dextran solution

	Infusion 6 per cent			Infusion 5 per cent		
	Before Hb_{a-d}	During Hb_{a-d}	Change	Before Hb_{a-dv}	During Hb_{a-d}	Change
M	+0 05	−0 19	−0 24	+0 01	−0 01	−0 02
SD	±0 14	−0 13	±0 12	±0 23	±0 17	±0 13
n	9	9	9	7	7	7
p			<0 001			>0 8

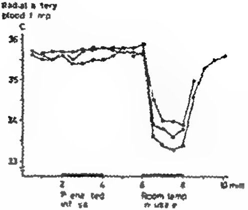

Fig 9 The radial artery blood temperature in three individuals at light exercise during infusions of a preheated (38 C) dye solution and a solution at room temperature

in the arterio-venous (a—v) haemoglobin concentration difference before and during infusion was calculated (Table 5) The (a—v) haemoblobin concentration differences did not differ significantly from zero either before or during infusion An infusion of 6 per cent dextran solution was accompanied by a significant change in the (a—v) difference indicating a small net transport of fluid from the extracellular space to the capillaries When 5 per cent dextran solution was infused however no significant change could be detected

As it would be physiologically undesirable to have the infusion alter the temperature of the arterial blood the syringe with dye solution was heated for at least 10 min in a thermostatically controlled waterbath until immediately before the start of infusion With the waterbath set at 38° C no change in radial artery blood temperature could be recorded during the subsequent infusion period The blood temperature fell substantially when a dye solution at room temperature was infused (Fig 9)

Routine procedure for determining brachial artery blood flow during exercise

Catheters are inserted into the brachial and radial arteries and a deep forearm vein The internal diameter of the tip of the brachial artery catheter should be 0 5 mm Indocyanine green solution (30 mg/l) in 5 per cent dextran solution is prepared and heated to 38° C A blood sample is drawn from the radial artery for determination of the pre infusion dye concentration The infusion (3+ ml/min) is started and after 1 min blood samples are drawn simultaneously from the radial artery and the deep vein for determination of dye concentration When duplicate blood samples have been obtained the infusion is stopped and the duration of the infusion period noted Recirculating dye is determined in a radial artery blood sample drawn 15—20 sec after the end of the infusion Dye concentrations are measured spectrophotometrically

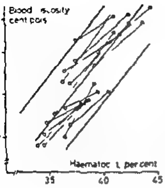

Fig 8 Effect of infusion (34 ml/min) on blood viscosity at a shear rate of 23 sec^{-1} The regression $\pm$ 2 SD of log viscosity on haematocrit is shown (log y = 0 0294x + 0 6049 SD 0 053 r = 0 85***) The thin lines join measurements in blood samples obtained before (●) and during infusion (○) in the same individual

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Dextran solutions of varying concentrations were infused for 1 min 15 sec into the brachial artery during light exercise (4 0 kpm/min) The haemoglobin concentration was measured in radial artery and deep venous blood immediately before the start and at the end of the infusion period The change

TABLE 5 Art[illegible]ous haemoglobin concentration difference (Hb_{a-dv} g per 100 ml) [illegible] during exercise in blood samples from the radial artery and a deep vein [illegible] and during infusion of 6 and 5 per cent dextran solution

	Infus[illegible]			Infusion 5 per cent		
	Before Hb_{a-d}	During Hb_{a-d}	Change	Before Hb_{a-d}	During Hb_{a-d}	Change
M	+0 05	[illegible]	−0 24	+0 01	−0 01	−0 02
SD	±0 14	±0 13	±0 12	±0 23	±0 17	±0 13
n	[illegible]	9	9	7	7	7
p			0 001			>0 8

Fig 11 Comparison between two determinations of blood flow during continuous exercise The observations were made with an interval of approximately 4 min The regression line (broken line y = 0 993x + 8 0 SD = 21 r = 0 98**) does not deviate significantly from the line of identity (p > 0 6)

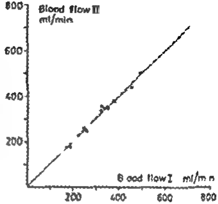
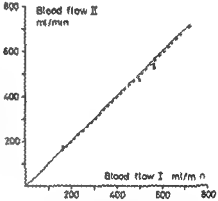


Fig 12 Comparison between two determinations of blood flow at repeated exercise The observations were made at the same work intensity separated by a rest period of at least 30 min The regression line (broken line y = 0 979x + 2 4 SD = 37 r = 0 97***) does not deviate significantly from the line of identity (p 0 4)



creased the resting blood flow more than tenfold Plethysmography recordings were obtained repeatedly for 2 min from the start of infusion after which deep and superficial venous blood samples were taken for dye determinations The mean value of the last two recordings immediately before sampling were used for the calculation of flow and comparison with the dye dilution findings The results are given in Fig 10 The calculated regression line did not deviate significantly from the line of identity

Reproducibility of blood flow determination

Duplicate determinations of blood flow were performed during continuous exercise according to the routine procedure in seven subjects on twenty six occasions The exercise was always of a steady state type as far as could be judged from the subjects statements The time interval between measurements was approximately 4 min The standard deviation for a single determination of flow under these conditions was found to be 18 ml/min within the

range 180—700 ml/min. The coefficient of variation for a single determination was 4.5 per cent (mean flow 391 ml/min). No significant systematic deviation was found for the difference between the first and second determination (Fig. 11).

The brachial artery flow was also measured in 15 subjects during repeated exercise with a 30 min interval of rest. These flow values were calculated from data from the experiments on the mixing of blood and dye. Sampling of blood from the radial artery, a deep and a superficial vein was started after 2 min infusion at 34 ml/min. When dye concentrations from all three sampling sites were used for the calculation of flow, the standard deviation for a single determination was found to be 22 ml/min. When blood flow was calculated from radial artery and deep vein concentrations only (Fig. 12), no significant change was found in the standard deviation (24 ml/min, $p > 0.4$) or flow values. The coefficient of variation for a single determination using measurements from these two sampling sites was 5.7 per cent (mean flow 424 ml/min).

Discussion

Procedure for the determination of brachial artery blood flow

In dye dilution studies of this type the indicator may be introduced either by a single injection or by continuous infusion. Single injection technique permits a smaller volume of the injectate and reduces the problem of recirculation. This procedure was tried in the present study and employed for the determination of circulation times in the forearm. For measuring blood flow, however, it was found that while mixing conditions were probably acceptable, the effect of the injection on blood flow was somewhat uncertain. The injection was found to cause vasodilatation in the forearm when performed at rest. Moreover, blood flow in the deep veins is usually intermittent during rhythmic exercise and continuous sampling is therefore occasionally impossible. Reproducible administration of dye was also a problem. When, instead, the indicator is infused continuously, the equilibrium concentration can be determined in 2 ml blood samples, which are easily obtained, and this procedure also proved easier to handle.

The inner diameter of the tip of the catheter used for the continuous infusion of indicator is probably critical for the induction of adequate mixing. In practice the tip should be as small as possible. In the present study an internal diameter of 0.3 mm and infusion at 34 ml/min proved a satisfactory combination. The routine procedure described involves a 1 min infusion period and sampling from only the radial artery and a deep vein. High bifurcation of the brachial artery — the predominant complication in this type of blood flow

measurement — is however detected even so Consequently it is unnecessary to take samples from a superficial vein as well particularly as this calls for twice as long an infusion period The background concentration of dye is interpolated from the dye concentrations measured in radial artery blood samples obtained immediately before and 15 sec after the infusion The timing of these samples should be as exact as possible since the background concentration changes rapidly

The procedure suggested for the determination of brachial artery blood flow during exercise requires a relative steady state during the 1 min infusion period If a higher frequency response for the measuring system is desired blood samples can be taken from the radial artery only after 20 sec infusion Moreover a continuous recording of the dye concentration in blood from the radial artery with a densitometer would give a continuous measurement of the blood flow for a short time

Mixing of dye and blood

The calculation of blood flow from dye concentration readings presupposes complete mixing of dye and arterial blood before the bifurcation of the brachial artery Since flow in the brachial artery is almost certainly laminar even during heavy exercise mixing must be induced by creating local turbulence at the point of injection This was attempted by Andres *et al* (1954) using an injector with a very small orifice In spite of the theoretical indications uniform distribution of dye in the forearm vascular system was not achieved more frequently than with ordinary low energy infusion Grace *et al* (1957) however stated that improved mixing conditions were found with high energy constant infusion into the thoracic aorta in man at infusion rates above 100 ml/min (internal diameter of catheter 0 5 mm kinetic energy/sec = 608 000 dynem/sec) The present findings are in agreement with this observation The uniformity of the dye distribution definitely improved with increasing infusion rate The lowest of the infusion rates tested that resulted in satisfactory mixing was 34 ml/min (kinetic energy/sec = 26 000 dynem/sec) Hydrodynamic considerations suggest that adequate mixing can be induced by an infusion of lower kinetic energy in the brachial artery than in the thoracic aorta owing to the smaller diameter of the former (Reynolds 1883)

Drastic disagreement between dye concentrations in blood from the radial artery a deep and a superficial vein was however found in 5 out of 34 subjects (17 per cent) during dye infusion The dye concentrations from different sampling points displayed a fixed relationship for each subject largely independent of variations in the infusion rate or work intensity This uneven dye distribution was ascribed to high anomalous bifurcation of the brachial

artery, in two of these subjects two separate arterial pulsations could be palpated in the antecubital fossa. Results from these five subjects have therefore been excluded. The incidence of this anomaly in man was stated by Quain (1844) to be 20 per cent. The presence of dual arterial supply to the forearm invalidates dye dilution techniques for the measurement of forearm blood flow (Andres *et al.* 1954) since the dye cannot then be completely mixed with the total arterial inflow. Careful palpation of the arteries of the arm in combination with manual compression may provide some indication of the arterial anatomy but the only certain way of excluding high bifurcation is a preliminary angiography.

The question arises as to what extent collateral arteries about the elbow contribute to forearm blood flow. Collateral arterial flow, although unlikely, may be relatively equally distributed to the radial, ulnar and interosseous arteries. Agreement between the concentrations in blood from the different sampling sites does not then conclusively exclude the presence of a significant collateral flow (Andres *et al.* 1954). However, an acceptable accordance was found between the blood flow as measured by venous occlusion plethysmography and by the indicator dilution procedure (Fig. 10). It is thus probable that the contribution of collaterals to forearm blood flow is very small.

Effect of infusion on blood flow

Forceful intraarterial injections have been reported to cause mechanical destruction of erythrocytes by a release of adenosine triphosphate and possibly other substances giving rise to vasodilatation (Fleisch 1937, Folkow 1952, Andres *et al.* 1954, Grace *et al.* 1957, Nilsson 1957). Andres *et al.* (1954) suggested that the rupture of erythrocytes during infusion is caused by the shearing stresses in the turbulent border layer close to the jet and that the kinetic energy per sec of the infusion determines the occurrence of haemolysis. Using jet injectors with an internal diameter of 25—50 μ the critical level of kinetic energy for erythrocyte destruction in the brachial artery at rest was found to be 9 000—34 000 dyncm/sec. This observation is confirmed in the present study. The forearm blood flow measured by plethysmography rose significantly after injections of saline (31 800 dyncm/sec) into the brachial artery at rest.

The effect of the infusion on brachial artery blood flow during exercise was evaluated from variations in venous oxygen saturation and haemoglobin concentration before and during infusion of dye. The oxygen consumption of muscle during steady state exercise has been found in animal experiments to remain constant during moderate changes in blood flow (Pappenheimer 1941, Stainsby and Otis 1964). Similarly moderate changes in coronary blood flow

do not influence the oxygen consumption of the working heart (Ross *et al* 1963) In these experiments flow changes were compensated for by alterations in the (a—v) oxygen difference In the present study infusion of dye at 34 ml/min (23 700 dynecm/sec) during exercise was found to elicit no significant change in either deep or superficial venous oxygen saturation This finding is compatible with the hypothesis that the infusion does not interfere with the blood flow except by adding itself to the preinfusion blood flow Furthermore the decrease in haemoglobin concentration during this type of infusion likewise indicated a simple addition of the infused solution to the initial blood flow within the flow range tested

On the other hand at the higher infusion rate of 52 ml/min (79 700 dynecm/sec) the venous oxygen saturation was significantly reduced especially at low work intensities A fraction of the preinfusion blood flow was thus probably replaced by the infusion making this infusion rate unsuitable for determinations of blood flow. The same situation presumably occurs during infusion of 34 ml/min at rest or during lighter exercise than was used in the present study Infusion of dye into the brachial artery at rest has been found to cause a marked vasodilatation when the kinetic energy is raised above 34 000 dynecm sec (Andres *et al* 1954) It is surprising that in the present study infusion at as much as 84 600 dynecm/sec during exercise was accompanied by a reduction of venous oxygen saturation i e a decrease of the preinfusion brachial artery blood flow This finding may reflect a changed influence during exercise of the vasodilating substances possibly released by haemolysis However the magnitude of the shearing stresses around the jet — causing destruction of erythrocytes during infusion — is related not only to the kinetic energy (as suggested by Andres *et al* 1954) but also and probably more directly to the linear velocity of the jet The present findings seem to support this view since the highest linear velocity used (430 cm sec catheter tip diameter 0 5 mm) was considerably lower than the velocity at the lowest critical infusion rate (1 600 cm/sec injector diameter 0 025 mm reported by Andres *et al* (1954)

Effect of infusion on the flow properties of blood

The question arises as to how the infusion at 34 ml/min during exercise is accomodated by the forearm vascular system It is hard to believe that this is accomplished by an active vasodilatation caused by destruction of erythrocytes during infusion (Andres *et al* 1954) so finely balanced as to cause simple addition of the infused solution to the initial blood flow

Infusion of 5 per cent dextran solution in this study was accompanied by a lowering of haematocrit which will tend to decrease the viscosity of whole blood (Rand *et al* 1964) However when blood is diluted with dextran (mean

catheter A deep venous blood sample was obtained immediately afterwards for pO_2 analysis. *During continued rhythmic exercise the deep venous catheter* was then withdrawn 3—5 cm. A new dye dilution curve was recorded and a blood sample obtained for pO_2 determination. In some cases dye dilution curves were also recorded when the deep venous catheter had been further withdrawn and the tip of the catheter could be palpated subcutaneously. Dye dilution curves were also recorded from superficial veins.

Series F In 15 subjects the brachial artery and a deep forearm vein were catheterized. After 8 min exercise at 10 kpm min dye dilution curves were recorded from the deep vein after the injection of dye (0 25 mg) into the brachial artery. A deep venous blood sample was obtained immediately afterwards for oxygen saturation analysis.

Methods

The concentration of indocyanine green in serum was measured spectrophotometrically at the point of maximal absorption (close to 805 mμ). A correction was made for recirculating dye by linear interpolation between the concentrations immediately before and after the infusion. The standard deviation for a single analysis of dye concentration in the range 0—2 mg/l was 0 10 mg/l. This value was calculated from 20 sets of duplicate blood samples obtained simultaneously. When the concentration of dye in venous samples during work exceeded the arterial concentration by less than 0 32 mg/l the difference was considered insignificant (confidence 99 per cent). The difference tolerated constitutes 2 6 per cent of the mean dye concentration of blood samples from deep veins during infusion at rest (12 1 mg/l).

Results

A Venous oxygen saturation — body heating

Rest

Ten experiments were performed. The oxygen saturation in blood samples from superficial and deep veins at rest with and without arterial occlusion at the wrist both before and during heating is given in Fig 13. The oxygen saturation was highly significantly lower in blood from deep veins after wrist occlusion than in blood from superficial veins both before and during body heating. In the majority of cases the oxygen saturation of blood from deep forearm veins was lower after wrist occlusion than before. The deep venous oxygen saturations with and without wrist occlusion were probably significantly higher during heating than before.

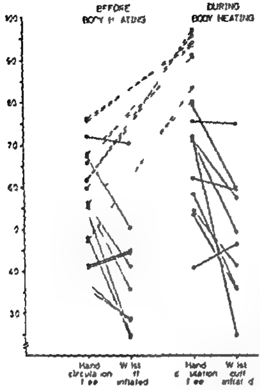


Fig. 13 Oxygen saturation recorded at rest in blood samples from deep (●——●) and superficial veins (○– – ○) before and during body heating with and without wrist occlusion

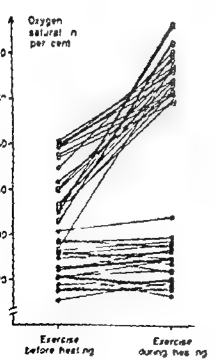


Fig. 14 Oxygen saturation recorded in ten experiments during exercise before and during body heating in blood samples from superficial veins (○——○) and from deep veins (●——●)

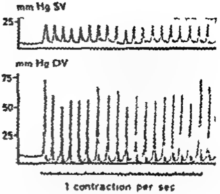


Fig 15 Pressure recordings from a deep (DV) and a superficial (SV) forearm vein at work intensity 4 4 kpm/min and 60 contractions per min The recordings were made consecutively

Exercise

Deep venous blood Fig 14 shows the variations in oxygen saturation measured before and during heating in blood samples from deep and superficial forearm veins during work (15 min) The oxygen saturation in deep venous blood was 22 5 ± 3 9 per cent during the first work period and 23 9 ± 4 3 per cent during body heating The difference is not significant The oxygen saturation does not differ significantly between blood samples obtained after 10 and 15 min The coefficient of variation for a single observation during exercise calculated from paired 10 and 15 min samples between the two work periods was 8 7 per cent (n = 10 mean 23 2 per cent oxygen saturation)

Superficial venous blood The oxygen saturation for superficial venous blood was 40 5 ± 7 3 per cent before heating and 65 4 ± 5 8 per cent during heating The difference is highly significant After the transition from rest to work the oxygen saturation in superficial venous blood had decreased by 22 5 ± 8 4 per cent in the experiments performed at ordinary room temperature The corresponding value for the experiments during body heating was 24 5 ± 8 2 per cent The difference is not significant

Skin temperature during work was recorded in 4 cases from the middle of the volar surface of the forearm the back of the hand and the palm The mean increase in skin temperature before and during heating was on the forearm 5 4° C (3 5—7 7°) on the back of the hand 7 8° (6 1—9 2°) and on the palm 8 3° (6 8—12 4°)

Pressure variations in superficial and deep veins in connection with work (4 4 kpm/min 60 contractions per min) were determined in 7 cases To ensure that the tip of the catheter was free blood sampling always preceeded the pressure recordings The pressure at rest was 4 7 ± 1 7 mm Hg in deep veins and 3 7 ± 2 7 mm Hg in superficial veins The difference is not significant During work the pressure increased during contractions to 56 6 ± 21 6 mm Hg over the

TABLE 6 Finding of contrast medium and dye in the catheterized deep vein and other deep and superficial veins after administration in the radial artery at rest and during work (4 4 kpm/min 60 contractions per min) in 10 subjects

	At rest					During exercise				
	Presence of contrast medium in			Presence of infused dye in		Presence of contrast medium in			Presence of infused dye in	
Subjects	Catheterized deep vein	Other deep vein	Catheterized sup vein	Deep vein	Sup vein	Catheterized deep vein	Other deep vein	Catheterized sup vein	Deep vein	Sup vein
T I	−	−	−	−	+	−	−	+	−	+
P O	−	−	[1]	+	+	−	−	[1]	−	+
P R	−	−	+			−	−	+		
L L	+		+	−	+	−	−	+	−	+
S C	−	−	−	+	+	−	−	+	−	+
S B	+		+	+	+	−	−	+	−	+
T J	−	−	−	−		−	−	−	−	[2]
F C	−	−	+	+	+	−	−	+	−	+
R N	−	+	+	+	+	[3]	−	+	−	+
N P	−	−	−	−	+	−	−	−	−	+

[1] Catheter not filled with contrast medium
[2] No sample obtained due to vasospasm
[3] Presence of contrast in deep vein doubtful in one series definitely negative in repeated experiments

resting level in deep veins and to 12 0 ± 4 2 mm Hg in superficial veins (Fig 15) The difference between the two types of veins is significant

B X-ray series

Ten experiments were performed The contrast medium injected into the radial artery at the wrist gave satisfactory filling of the forearm veins via the hand veins and permitted differentiation between superficial and deep veins in all cases The films were studied for the presence of contrast medium both in the catheterized superficial and deep veins and in other veins The injection of contrast medium at rest usually elicited transitory pain in the hand and some times also in the lower forearm The intensity of the pain was individual but was most intense in a few cases with reflux of the contrast to the forearm

a b

Fig. 16 (a) Contrast medium may be seen in both deep and superficial veins after injection at rest into the radial artery at the wrist. The two venous catheters are visualized by contrast filling with their tips indicated by arrows.

(b) No contrast in the deep veins after injection into the radial artery during exercise (4.4 kpm/min, 60 contractions per min). Exposure 6 sec after the start of injection.

arteries. The appearance of contrast in the forearm veins was then delayed. There was no significant reflux upon repeated slower injection of contrast about 15 min later, combined if necessary with manual compression of the radial and ulnar arteries immediately proximal to the wrist. During work there was no reflux in any case and the pain on injection was mild. The veins always filled well and considerably more rapidly than at rest.

Rest

The position of the catheter in the deep vein could be confirmed in all ten cases. The results of the x-ray investigation and the dye infusion are given in Table 6. At rest, deep veins filled in 3 cases, 2 of which were the catheterized deep vein. In these 3 and a further 3 cases there was dye in the blood sample from the catheterized deep vein. A large number of superficial veins always filled, including the catheterized vein in 5 cases. In one case contrast leakage prevented the location of the superficial catheter and in one case vasospasm of the catheterized superficial vein made it impossible to obtain blood samples. Dye was found in the samples from the superficial veins in all experiments.

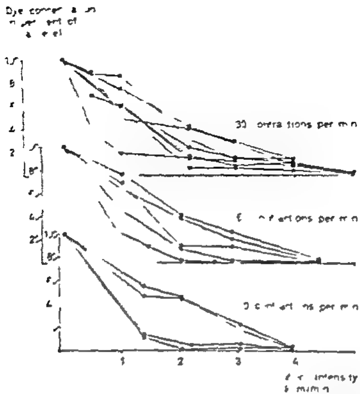


Fig. 17. Concentration of dye in deep venous blood during exercise of different intensities. The dye was infused continuously into the radial artery at rest and during exercise. The concentration during exercise is expressed as a percentage of the concentration recorded during infusion at rest.

Exer

As a … no contrast was detected in the deep veins (Table 6, Fig. 16). In one case … a minute amount of contrast medium appeared in the catheterized vein … during work in one series of exposures. Repeated injection did not cau… … vein to fill again. In all nine cases in which blood samples were obtained from the deep veins during dye infusion the blood was free from significant concentrations of dye.

(Dye infusion — varying work intensity

The concentrations of indocyanine green in blood samples from deep forearm veins during the infusion of dye in the radial artery at work of varying intensity is shown in Fig. 17. The concentrations declined with increasing

Table 7 Individual values for the mean transit time (sec) during exercise (10 kpm/min) from the brachial artery to a deep forearm vein and the pO (mm Hg) of deep venous blood Measurements performed with the venous catheter advanced as distally as possible and then repeated with the catheter withdrawn 3—5 cm proximally

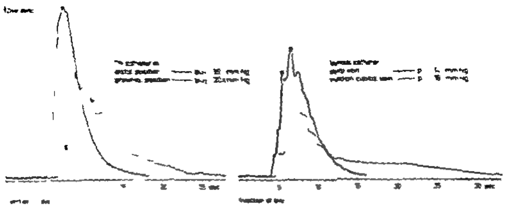
Subject	Mean transit time sec			pO_2 mm Hg		
	Catheter tip in			Catheter tip in		
	distal position	proximal position	Change	distal position	proximal position	Change
1	8 4	9 2	+0 8	23 0	23 0	±0
2	8 4	10 ?	−1 8	18 5	18 5	±0
3	6 7	8 3	+0 6	14 0	15 0	+1 0
4	8 2	10 6	+2 4	2? 0	20 5	−1 5
5	8 0	9 2	−1 2	13 0	12 0	−1 0
6	6 8	7 9	+1 1	14 0	13 5	−0 5
7	9 2	12 1	+?9	19 ?	20 0	−0 5

intensity of work and no significant concentrations of dye were found at work intensities of approximately 4 kpm/min or more at contraction frequencies of 60 and 90 per min During work at a frequency of 30 contractions per min and 4 kpm/min there was a low but significant concentration of dye in the deep venous blood, but at 5 kpm/min no dye could be detected

The dye concentration in blood from superficial veins showed irregular variations Usually the concentration decreased during work though it did rise in some cases, 30 per cent of the rest value was the lowest concentration measured during work

D pO_2 — varying catheter position

Deep veins The deep venous catheter was first advanced as far as possible and then withdrawn 3—5 cm In both catheter positions dye dilution curves were recorded and pO_2 was determined in blood samples obtained through the catheter This series covers 7 experiments and the results are presented in Table 7 and Fig 18a The pO of deep venous blood did not differ significantly ($p > 0.3$) with the catheter in the distal and proximal positions The mean transit time as determined from the dye dilution curve invariably increased when the catheter was withdrawn ($p < 0.01$) In all cases the shape of the curve was altered the peak usually being lower and the base broader (Fig 18a) When plotted in a semilogarithmic system the downslope of the curves



a b

Fig. 18. Dye dilution curves recorded after the injection of 0.25 mg dye into the brachial artery.

a. Dilution curves when sampling with the catheter advanced as far as possible (unbroken line) and withdrawn 3—5 cm (broken line).

b. Dilution curves with the venous catheter in a deep vein (unbroken line) and after withdrawal until its tip lay in the median cubital vein, proximal to the confluence of a superficial vein (broken line). The pO_2-values for blood obtained with the venous catheter in different positions are indicated.

appeared monoexponential, with a steeper slope for the catheter in the distal position.

In three cases dye dilution curves were also recorded with the catheter withdrawn until the tip was palpable subcutaneously proximally to the confluence of a superficial vein. In these cases a rise in venous pO_2 was recorded and the dye curves showed an increased passage time (Fig. 18b). A semilogarithmic plot of the downslope of these curves indicated at least two different slopes.

Superficial veins. Dye dilution curves were recorded from superficial veins during exercise at 10 kpm/min in 7 experiments. Some of the curves were markedly asymmetrical, with one or several peaks, while others were smooth but showed low peaks and long mean transit times (Fig. 19).

E. Oxygen saturation — mean transit time

Dye dilution curves were recorded from deep veins after ca. 8 min exercise at 10 kpm/min in [illegible] subjects. The dye was injected into the brachial artery. After the dilution curve had been registered, blood samples were drawn for oxygen saturation analysis. The mean transit time was determined from the curves. The deep venous oxygen saturation was 31.5 ± 7.4 per cent and the mean transit time 8.[illegible] ± 0.8 sec. The correlation between deep venous oxygen

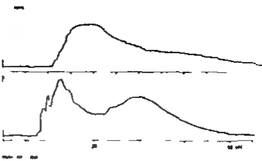


Fig 19 Dye dilution curves for superficial forearm veins after the injection of dye (0 25 mg) into the brachial artery Note the late peak of the lower curve indicating long transit times in a large part of the vascular bed drained

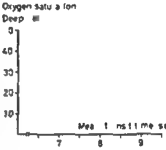


Fig 20 Oxygen saturation in blood samples obtained from deep veins in 12 subjects during exercise (10 kpm/min) The values are plotted as a function of the corresponding mean transit times determined from dye dilution curves No correlation was found (r = 0 09)

saturation and mean transit time (Fig 20) was not significant (r = 0 09) The downslope of the dilution curves was in all cases monoexponential in a semilogarithmic system

Discussion

Body heating is accompanied by an increased blood flow in the hand (Lewis and Pickering 1931 Gibbon and Landis 1932) and in the skin of the forearm (Roddie Shepherd and Whelan 1956 Edholm Fox and Macpherson 1956) while muscle blood flow appears to remain unchanged (McGirr 1952 Barcroft *et al* 1955) The increase in the blood flow of the hand is probably caused by release of vasoconstrictor tone In addition to this mechanism there may be an active vasodilatation in the forearm skin (Roddie *et al* 1957)

During forearm exercise the oxygen saturation recorded in deep venous blood remained unchanged at an ordinary room temperature and during body heating This probably indicates that the superficial venous blood does not contaminate the deep venous system during this type of work Certain conditions must be fulfilled however for this statement to be valid The arterial oxygen saturation is presumed to remain unchanged This has been shown to be the case both during exercise (Holmgren and Linderholm 1958 Pernow and Wahren 1962) and during body heating (Roddie *et al* 1956) It is also presupposed that muscle blood flow and metabolism are the same during both

work periods At rest forearm muscle metabolism (Koddie *et al* 1956) and blood flow (McGirr 1952, Barcroft *et al* 1955) appear to remain unchanged with body heating and it seems likely that this is also the case during exercise of this type

In series B no significant amount of contrast was detected in the deep veins of the forearm after injection into the radial artery during exercise in all but one case although the superficial veins were always clearly visible Moreover, the exception was a doubtful case and no contrast was found in the deep vein after renewed injection at rest on the other hand, the study was positive as regards both contrast and dye it is possible that this subject did not work satisfactorily during the first contrast injection

Dye dilution curves for the deep forearm veins during exercise always showed a monoexponential downslope suggesting that only one type of flow was present in the vascular bed sampled When on the other hand the catheter was withdrawn until the tip lay proximal to the confluence of a superficial vein at least two different slopes were found It is therefore likely that the samples from the deep veins did not contain superficial venous blood

The results of series A B and E thus agree well and indicate that superficial venous blood from the hand and the forearm superficial tissue does not pass to the deep venous system during exercise of this type At rest however indicator infused or injected into the radial artery (series B) was subsequently observed in the deep veins It appears that the circulatory adjustment occurs gradually with increasing work intensity and that it is to some extent dependent on the frequency of contraction (cf Fig 17) With a work intensity of approximately 4 kpm/min and frequencies of 60 and 90/min no significant dye concentration could be demonstrated in deep venous blood whereas there was a small but significant concentration at 30/min

The gradual fall in the dye concentration with increasing work intensity can partly be explained by the increased blood flow through the working muscle However the initial resting dye concentration was high in all cases (mean 12 1 mg/l) and a more than thirtyfold mean increase in the deep venous blood flow from the resting value would be needed to account for the disappearance of dye solely by dilution Since the exercise was of a steady state type and of low to moderate work intensity it seems that other factors besides dilution may be of importance

When the subject grasps the two handles of the hand ergometer and performs the contraction the proximal handle presses against the thenar eminence and the ulnar part of the palm while the other handle rests against the middle phalanges During contraction most of the veins passing through the proximal part of the palm are probably wholly or partly compressed These vessels

connect with the deep system in the forearm as *venae comitantes* to the radial and ulnar arteries It is also probable that the veins to the back of the hand at the level of the metacarpophalangeal joints are not obstructed during contraction As a result the hand effluent blood may be redistributed via these vessels to the back of the hand and to the superficial venous system of the forearm Judging from the strongly increased pressure in the deep venous system during the contractions the flow of blood between the two venous systems of the forearm is from the deep towards the superficial veins

The mean transit time for dye from the brachial artery to the deep veins during exercise increased significantly when the catheter was withdrawn 3—5 cm Moreover, the shape of the curve changed and the downslope became less steep The exercise was of a steady state type and blood flow may be assumed to have remained the same Hence the results indicate that a larger vascular bed was sampled with the catheter in the proximal position Nevertheless deep venous pO_2-values did not change significantly suggesting that there is little difference in the pO_2 of blood from different parts of the deep venous system This finding is further supported by the lack of correlation between deep venous oxygen saturation and mean transit time in the interindividual study since mean transit time may be used as an index of the catheter s position

The shape of the downslope of a dye dilution curve of this type is determined by the relationship between flow and volume in the different parts of the vascular system traversed by the indicator As mentioned above dye curves recorded from deep forearm veins during exercise showed a largely monoexponential downslope In this respect the resolution of the measuring system is limited by the distortion of the curves due to effects of the catheter and the recording equipment All downslopes appeared however linear in a semilogarithmic system to within 2—3 per cent of the peak dye concentration value This indicates that all parts of the forearm vascular system drained by the deep vein showed the same relationship between flow and vascular volume i e that the perfusion per unit vascular volume was identical in all parts With a reservation for the unlikely possibility that variations in flow within the region were balanced by equal changes in vascular volume this finding supports the view that the perfusion per unit tissue was the same in all parts of the region under study This may seem surprising considering that the flexor muscles probably work much more than other forearm muscle groups during this type of exercise Most likely the uniform homogenous flow passes through the active forearm muscles while inactive muscle groups and other deep forearm structures may either not be drained by the vascular bed sampled with the catheter or have an insignificant blood flow in comparison with that of the active muscles

CHAPTER V

Circulatory and Metabolic Response to Different Types of Rhythmic Isotonic Exercise

Subjects

Results presented in this chapter are based on data from 30 male volunteers aged 20—51 (mean 30.3 years); fourteen were firemen and sixteen were students. All subjects had had regular health controls and were in good health at the time of the investigation.

Procedure

The subjects came fasting to the laboratory in the morning. After the various dimensions of the arm had been measured, teflon catheters were inserted into the brachial and radial arteries, a deep and a superficial vein of one forearm. The right arm was generally used except in left handed individuals. The resting blood flow of the forearm and hand was then determined by venous occlusion plethysmography. Blood samples were obtained from the radial artery, a deep and a superficial vein for analysis of oxygen saturation, haemoglobin and lactate concentrations. The deep venous samples drawn at rest were always obtained after wrist occlusion for more than 3 min.

The subjects then performed three periods of rhythmic exercise on a hand ergometer (Fig. 1). Each period lasted for 12 min and was followed by a rest interval of 35—45 min. The work intensity was set at 5, 10 and 15 kpm/min. The sequence of the two exercise periods with the lower work intensities was randomized (16 subjects starting with 5 and 14 with 10 kpm/min).

The study was arranged in four different series of 10 experiments each. In Series 1, 2 and 3 the experiments involved varying types of exercise but the same total work per unit time (5, 10 and 15 kpm/min). In Series 1 the work intensity was varied by increasing the *load per contraction* (2.8, 5.6 and 8.3 kp) while the [illegible] of contraction was kept at 30 mm and the frequency of contraction at 60[illegible] In Series 2 three different *frequencies* were used (30, 60 and 90/min [illegible] the load and the path of contraction were kept constant at 5.6 kp and 3[illegible] respectively. In Series 3 the *path of contraction* was varied (15, 30 and 45 [illegible] the load (5.6 kp) and the frequency of contraction (60/min) being kep[illegible] tant. The exercise at 10 kpm/min was thus identical in all three series [illegible] bjects were represented twice and one three times in Series 1—3. Resul[illegible] duplicate experiments on the same individuals have, how

Fig 21 The symbols used for denoting the duration of the different phases of contraction and relaxation during exercise

ever been excluded in the presentation of data obtained at rest and in analyses of the combined material at 10 kpm/min Results from seven other identical experiments at 10 kpm/min in other subjects were used instead to bring up the total to thirty different subjects The results of the experiments at 10 kpm/min and the corresponding measurements at rest were subjected to multiple regression analysis All significant correlations and some others of special interest are presented below

In Series 4 the subjects carried out the exercise at 10 kpm min twice once with the forearm horizontal and once with the arm vertically elevated perpendicular to the rest of the body The hand ergometer was fixed to a stand adjusted to the appropriate height The experiments in Series 4 were conducted together with some of those in Series 2 and 3

Infusion of dye for blood flow determination and blood sampling was identical in all four series After 4 8 and 12 min exercise blood flow was determined with the routine procedure described above (see page 25) On the same occasions blood samples were taken from the radial artery and a deep vein for the determination of oxygen saturation haemoglobin and lactate concentrations Lactate and haemoglobin concentrations were analyzed in the same samples as dye concentration and were corrected for dilution by the infused solution Heart rate and the time course of contraction and relaxation were also determined after 4 8 and 12 min exercise T_1 was used to denote the time from the start of movement until completion of the path of contraction T_2 the duration of isometric contraction with the handles compressed and T_3 the time taken by the spring to return to its initial position T_4 indicates the time from the end of one relaxation till the start of the following contraction T_5 is the sum of $T_1 + T_2 + T_3$ while T_6 is the sum of $T_1 + T_2$ (cf Fig 21) The means for 10 registered cycles were computed for the registrations at 4 8 and 12 min during each exercise period

Brachial artery blood pressure at rest was measured with the arm both horizontal and vertical The intercondylar line of the humerus was used as reference level

Procedure

Series A During the first part of the experiment the room temperature varied between 17 and 19 C The subjects were lightly clothed with their torsos bare Catheters were introduced into a deep and a superficial vein, after which blood samples for determination of oxygen saturation were obtained with the hand circulation free and with arterial occlusion at the wrist Next, rhythmic exercise was performed for 15 min (1·4 kpm/min 60 contractions per min), blood samples being taken repeatedly during exercise for analysis of oxygen saturation A heat cupboard with a fan was then placed over the thorax and the subject was warmed up for 30 – 50 min with 40° C air The previous experimental procedure was then repeated during continued heating

Series B After catheters had been introduced into a deep and a superficial vein and into the radial artery 8 of the 10 subjects were given 5 ml Baralgin® i v to prevent vasospasm during the course of the experiment A contrast medium (4—10 ml 45 per cent Urografin sodium and methylglucamine salts of diatrizoate Schering) was injected by hand into the radial artery at an even rate over a period of 3—10 sec Beginning 3—7 5 sec after the start of injection 10 films were exposed at a rate of 1 per sec The exposures were made in two planes (frontal and side) at rest and during rhythmic work (4 4 kpm/min 60 contractions per min) During exercise the injection was made 3—4 min after the start of the work period The vein catheters were filled with contrast immediately before the exposures to facilitate identification on the films

In 9 cases after the x ray investigation indocyanine green solution (0 1 mg/ml) was infused at a constant rate (3 ml/min) for 2 min into the radial artery Duplicate blood samples were then taken from superficial and deep [illegible] veins at rest and 3—4 min after the beginning of exercise

[illegible] After the introduction of catheters into a deep and a superficial vein [illegible] the radial artery indocyanine green solution (0 1 mg/ml) was infused [illegible] at a constant rate in the radial artery in connection with work of [illegible] intensities Infusion of dye sampling and analysis were carried out as in [illegible] The intensity of work was varied either by changing the work per [illegible] by altering the spring tension or by changing the frequency of contraction The frequencies used were 30 60 and 90 contractions per min with [illegible] kpm min at the first and 1—4 kpm/min at the other frequencies

Series D The [illegible] artery a deep and a superficial vein were catheterized The deep venous catheter was advanced as far as possible Exercise at 10 kpm/min was then started Indocyanine green (0 25 mg) was injected after 6—8 min exercise into the brachial artery and the resultant dye dilution curve was recorded with a cuvette densitometer sampling through the deep venous

catheter. A deep venous blood sample was obtained immediately afterwards for pO_2 analysis. During continued rhythmic exercise the deep venous catheter was then withdrawn 3—5 cm. A new dye dilution curve was recorded and a blood sample obtained for pO_2 determination. In some cases dye dilution curves were also recorded when the deep venous catheter had been further withdrawn and the tip of the catheter could be palpated subcutaneously. Dye dilution curves were also recorded from superficial veins.

Series E. In 15 subjects the brachial artery and a deep forearm vein were catheterized. After [illegible] min exercise at 10 kpm/min dye dilution curves were recorded from the deep vein after the injection of dye (0.25 mg) into the brachial artery. A deep venous blood sample was obtained immediately afterwards for oxygen saturation analysis.

Methods

The concentration of indocyanine green in serum was measured spectrophotometrically at the point of maximal absorption (close to 805 mμ). A correction was made for recirculating dye by linear interpolation between the concentrations immediately before and after the infusion. The standard deviation for a single analysis of dye concentration in the range 0—2 mg/l was 0.10 mg/l. This value was calculated from 20 sets of duplicate blood samples obtained simultaneously. When the concentration of dye in venous samples during work exceeded the arterial concentration by less than 0.32 mg/l the difference was considered insignificant (confidence 99 per cent). The difference tolerated constitutes 2.6 per cent of the mean dye concentration of blood samples from deep veins during infusion at rest (12.1 mg/l).

Results

A. Venous oxygen saturation — body heating

Rest

Ten experiments were performed. The oxygen saturation in blood samples from superficial and deep veins at rest with and without arterial occlusion at the wrist, both before and during heating, is given in Fig. 13. The oxygen saturation was highly significantly lower in blood from deep veins after wrist occlusion than in blood from superficial veins both before and during body heating. In the majority of cases the oxygen saturation of blood from deep forearm veins was lower after wrist occlusion than before. The deep venous oxygen saturations with and without wrist occlusion were probably significantly higher during heating than before.

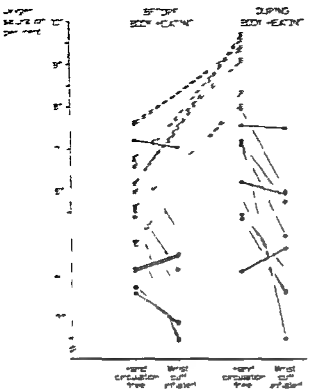


Fig. 13. Oxygen saturation recorded at rest in blood samples from deep ●——● and superficial veins ○– – –○ before and during body heating with and without wrist occlusion.

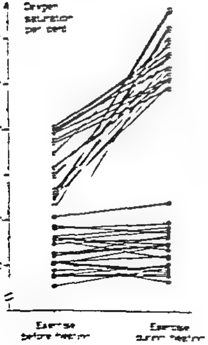
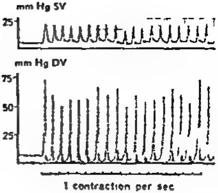


Fig. 14. Oxygen saturation recorded in ten experiments during exercise before and during body heating in blood samples from superficial veins ○——○ and from deep veins ●——●

Fig 15 Pressure recordings from a deep (DV) and a superficial (SV) forearm vein at work intensity 4 4 kpm/min and 60 contractions per min The recordings were made consecutively



Exercise

Deep venous blood Fig 14 shows the variations in oxygen saturation measured before and during heating in blood samples from deep and superficial forearm veins during work (15 min) The oxygen saturation in deep venous blood was 22 5 $\pm$ 3 9 per cent during the first work period and 23 9 $\pm$ 4 3 per cent during body heating The difference is not significant The oxygen saturation does not differ significantly between blood samples obtained after 10 and 15 min The coefficient of variation for a single observation during exercise calculated from paired 10 and 15 min samples between the two work periods was 8 7 per cent (n = 10 mean 23 2 per cent oxygen saturation)

Superficial venous blood The oxygen saturation for superficial venous blood was 40 5 $\pm$ 7 3 per cent before heating and 65 4 $\pm$ 5 8 per cent during heating The difference is highly significant After the transition from rest to work the oxygen saturation in superficial venous blood had decreased by 22 5 $\pm$ 8 4 per cent in the experiments performed at ordinary room temperature The corresponding value for the experiments during body heating was 24 5 $\pm$ 8 2 per cent The difference is not significant

Skin temperature during work was recorded in 4 cases from the middle of the volar surface of the forearm the back of the hand and the palm The mean increase in skin temperature before and during heating was on the forearm 5 4° C (3 5—7 7°) on the back of the hand 7 8° (6 1—9 2°) and on the palm 8 3° (6 8—12 4°)

Pressure variations in superficial and deep veins in connection with work (4 4 kpm/min 60 contractions per min) were determined in 7 cases To ensure that the tip of the catheter was free blood sampling always preceeded the pressure recordings The pressure at rest was 4 7 $\pm$ 1 7 mm Hg in deep veins and 3 7 $\pm$ 1 7 mm Hg in superficial veins The difference is not significant During work the pressure increased during contractions to 56 6 $\pm$ 21 6 mm Hg over the

[illegible] [illegible] deep [illegible] other deep [illegible] in the radial artery at rest and during [illegible]

[illegible]	[illegible]					[illegible]				
	[illegible]			[illegible]		[illegible]			[illegible]	
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
T J	—	—	—	—	—	—	—	—	—	—
[illegible]	—	—		—	—	—	—		—	—
P P	—	—	—			—	—	—		
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[illegible]	—	—	—	—	—	—	—	—	—	—
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[illegible]	—	—	—	—	—	—	—	—	—	—

[illegible]

[illegible]

[illegible]

Fig 16 (a) Contrast medium may be seen in both deep and superficial veins after injection at rest into the radial artery at the wrist The two venous catheters are visualized by contrast filling with their tips indicated by arrows

(b) No contrast in the deep veins after injection into the radial artery during exercise (4 4 kpm/min 60 contractions per min) Exposure 6 sec after the start of injection

a b

arteries The appearance of contrast in the forearm veins was then delayed There was no significant reflux upon repeated lower injection of contrast about 15 min later combined if necessary with manual compression of the radial and ulnar arteries immediately proximal to the wrist During work there was no reflux in any case and the pain on injection was mild The vein always filled well and considerably more rapidly than at rest

Rest

The position of the catheter in the deep vein could be confirmed in all ten cases The results of the x ray investigation and the dye infusion are given in Table [redacted] At rest deep veins filled in 3 cases [redacted] of which were the catheterized deep vein In these 3 and a further 3 cases there was dye in the blood sample from the catheterized deep vein A large number of superficial veins always filled including the catheterized vein in 5 cases In one case contrast leakage prevented the location of the superficial catheter and in one case vasospasm of the catheterized superficial vein made it impossible to obtain blood samples Dye was found in the samples from the superficial veins in all experiments

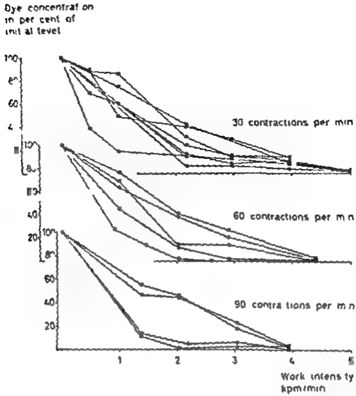


Fig 17 Concentration of dye in deep venous blood during exercise of different intensities The dye was infused continuously into the radial artery at rest and during exercise The concentration during exercise is expressed as a percentage of the concentration recorded during infusion at rest

Exercise

As a rule no contrast was detected in the deep veins (Table 6 Fig 16) In one case however a minute amount of contrast medium appeared in the catheterized deep vein during work in one series of exposures Repeated injection did not cause the vein to fill again In all nine cases in which blood samples were obtained from the deep veins during dye infusion the blood was free from significant concentrations of dye

C Dye infusion — varying work intensity

The concentration of indocyanine green in blood samples from deep forearm veins during the infusion of dye in the radial artery at work of varying intensity is shown in Fig 17 The concentrations declined with increasing

TABLE 7 Individual values for the mean transit time (sec) during exercise (10 kpm/min) from the brachial artery to a deep forearm vein and the pO_2 (mm Hg) of deep venous blood. Measurements performed with the venous catheter advanced as distally as possible and then repeated with the catheter withdrawn 3—5 cm proximally

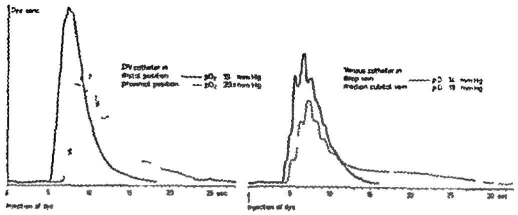
	Mean transit time sec			pO_2 mm Hg		
	Catheter tip in			Catheter tip in		
Subject	distal position	proximal position	Change	distal position	proximal position	Change
1	8.4	9.2	+0.8	23.0	23.0	±0
2	8.4	10.2	+1.8	18.5	18.5	±0
3	6.7	8.3	+0.6	14.0	15.0	−1.0
4	8.2	10.6	+2.4	22.0	20.5	−1.5
5	8.0	9.2	+1.2	13.0	12.0	−1.0
6	6.8	7.9	+1.1	14.0	13.5	−0.5
7	9.2	12.1	+2.9	19.5	20.0	+0.5

intensity of work and no significant concentrations of dye were found at work intensities of approximately 4 kpm/min or more at contraction frequencies of 60 and 90 per min. During work at a frequency of 30 contractions per min and 4 kpm/min, there was a low but significant concentration of dye in the deep venous blood but at 5 kpm/min no dye could be detected.

The dye concentration in blood from superficial veins showed irregular variations. Usually the concentration decreased during work, though it did rise in some cases. 30 per cent of the rest value was the lowest concentration measured during work.

D. pO_2 — varying catheter position

Deep veins. The deep venous catheter was first advanced as far as possible and then withdrawn 3—5 cm. In both catheter positions dye dilution curves were recorded and pO_2 was determined in blood samples obtained through the catheter. This series covers 7 experiments and the results are presented in Table 7 and Fig. 18a. The pO_2 of deep venous blood did not differ significantly ($p > 0.3$) with the catheter in the distal and proximal positions. The mean transit time as determined from the dye dilution curve invariably increased when the catheter was withdrawn ($p < 0.01$). In all cases the shape of the curve was altered, the peak usually being lower and the base broader (Fig. 18a). When plotted in a semilogarithmic system the downslope of the curves



a b

Fig 18 Dye dilution curves recorded after the injection of 0 25 mg dye into the brachial artery

(a) Dilution curves when sampling with the catheter advanced as far as possible (unbroken line) and withdrawn 3—5 cm (broken line)

(b) Dilution curves with the venous catheter in a deep vein (unbroken line) and after withdrawal until its tip lay in the median cubital vein proximal to the confluence of a superficial vein (broken line) The pO_2-values for blood obtained with the venous catheter in different positions are indicated

appeared monoexponential with a steeper slope for the catheter in the distal position.

In three cases dye dilution curves were also recorded with the catheter withdrawn until the tip was palpable subcutaneously proximally to the confluence of a superficial vein In these cases a rise in venous pO was recorded and the dye curves showed an increased passage time (Fig 18b) A semilogarithmic plot of the downslope of these curves indicated at least two different slopes

Superficial veins Dye dilution curves were recorded from superficial veins during exercise at 10 kpm/min in 7 experiments Some of the curves were markedly asymmetrical, with one or several peaks while others were smooth but showed low peaks and long mean transit times (Fig 19)

E Oxygen saturation — mean transit time

Dye dilution curves were recorded from deep veins after ca 8 min exercise at 10 kpm/min in 12 subjects The dye was injected into the brachial artery After the dilution curves had been registered blood samples were drawn for oxygen saturation analysis The mean transit time was determined from the curves. The deep venous oxygen saturation was 31 3 $\pm$ 7 4 per cent and the mean transit time 8 0 $\pm$ 0 8 sec The correlation between deep venous oxygen

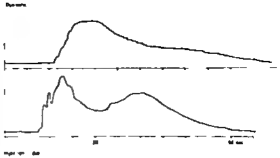


Fig 19 Dye dilution curves for superficial forearm veins after the injection of dye (0 25 mg) into the brachial artery Note the late peak of the lower curve indicating long transit times in a large part of the vascular bed drained

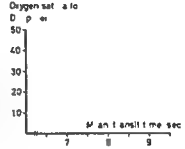


Fig 20 Oxygen saturation in blood samples obtained from deep veins in 12 subjects during exercise (10 kpm/min) The values are plotted as a function of the corresponding mean transit times determined from dye dilution curves No correlation was found (r = 0 09)

saturation and mean transit time (Fig 20) was not significant (r = 0 09) The downslope of the dilution curves was in all cases monoexponential in a semilogarithmic system

Discussion

Body heating is accompanied by an increased blood flow in the hand (Lewis and Pickering 1931 Gibbon and Landis 1932) and in the skin of the forearm (Roddie Shepherd and Whelan 1956 Edholm, Fox and Macpherson 1956) while muscle blood flow appears to remain unchanged (McGirr 1952 Barcroft *et al* 1955) The increase in the blood flow of the hand is probably caused by release of vasoconstrictor tone In addition to this mechanism there may be an active vasodilatation in the forearm skin (Roddie *et al* 1957)

During forearm exercise, the oxygen saturation recorded in deep venous blood remained unchanged at an ordinary room temperature and during body heating This probably indicates that the superficial venous blood does not contaminate the deep venous system during this type of work Certain conditions must be fullfilled however for this statement to be valid The arterial oxygen saturation is presumed to remain unchanged This has been shown to be the case both during exercise (Holmgren and Linderholm 1958 Pernow and Wahren 1962) and during body heating (Roddie *et al* 1956) It is also presupposed that muscle blood flow and metabolism are the same during both

CHAPTER V

Circulatory and Metabolic Response to Different Types of Rhythmic Isotonic Exercise

Subjects

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Procedure

The subjects came fasting to the laboratory in the morning. After the various dimensions of the arm had been measured, teflon catheters were inserted into the brachial and radial arteries, a deep and a superficial vein of one forearm. The right arm was generally used except in left handed individuals. The resting blood flow of the forearm and hand was then determined by venous occlusion plethysmography. Blood samples were obtained from the radial artery, a deep and a superficial vein for analysis of oxygen saturation, haemoglobin and lactate concentrations. The deep venous samples drawn at rest were always obtained after wrist occlusion for more than 3 min.

The subjects then performed three periods of rhythmic exercise on a hand ergometer (Fig. 1). Each period lasted for 12 min and was followed by a rest interval of 35—45 min. The work intensity was set at 5, 10 and 15 kpm/min. The sequence of the two exercise periods with the lower work intensities was randomized: 16 subjects starting with 5 and 14 with 10 kpm/min.

The study was arranged in four different series of 10 experiments each. In series 1, 2 and 3 the experiments involved varying types of exercise but the same total work per unit time (5, 10 and 15 kpm/min). In Series 1 the work intensity was varied by increasing the *load per contraction* (2.8, 5.6 and 8.3 kp) while the path of contraction was kept at 30 mm and the frequency of contraction at 60/min. In Series 2, three different *frequencies* were used (30, 60 and 90/min) while the load and the path of contraction were kept constant at 5.6 kp and 30 mm respectively. In Series 3 the *path of contraction* was varied (15, 30 and 45 mm), the load (5.6 kp) and the frequency of contraction (60/min) being kept constant. The exercise at 10 kpm/min was thus identical in all three series. Six subjects were represented twice and one three times in Series 1—3. Results from duplicate experiments on the same *individuals have* *how*

Fig. 21. The symbols used for denoting the duration of the different phases of contraction and relaxation during exercise

ever been excluded in the presentation of data obtained at rest and in analyses of the combined material at 10 kpm/min. Results from seven other identical experiments at 10 kpm/min in other subjects were used instead to bring up the total to thirty different subjects. The results of the experiments at 10 kpm/min and the corresponding measurements at rest were subjected to multiple regression analysis. All significant correlations and some others of special interest are presented below.

In Series 4 the subjects carried out the exercise at 10 kpm/min twice, once with the forearm horizontal and once with the arm vertically elevated perpendicular to the rest of the body. The hand ergometer was fixed to a stand adjusted to the appropriate height. The experiments in Series 4 were conducted together with some of those in Series 2 and 3.

Infusion of dye for blood flow determination and blood sampling was identical in all four series. After 4, 8 and 12 min exercise blood flow was determined with the routine procedure described above (see page 25). On the same occasions blood samples were taken from the radial artery and a deep vein for the determination of oxygen saturation, haemoglobin and lactate concentrations. Lactate and haemoglobin concentrations were analyzed in the same samples as dye concentration and were corrected for dilution by the infused solution. Heart rate and the time course of contraction and relaxation were also determined after 4, 8 and 12 min exercise. T_1 was used to denote the time from the start of movement until completion of the path of contraction, T_2 the duration of isometric contraction with the handles compressed and T_3 the time taken by the spring to return to its initial position. T_4 indicates the time from the end of one relaxation till the start of the following contraction. T is the sum of $T_1 + T_2 + T_3$ while T_6 is the sum of $T_1 + T$ (cf. Fig. 21). The means for 10 registered cycles were computed for the registrations at 4, 8 and 12 min during each exercise period.

Brachial artery blood pressure at rest was measured with the arm both horizontal and vertical. The intercondylar line of the humerus was used as reference level.

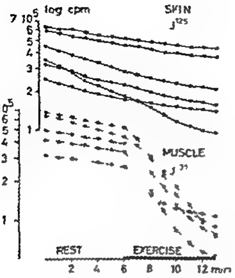

Fig 22 Clearance of J^{125} and J^{131} antipyrine after intracutaneous (●———●) and intramuscular (● — — — ●) injections at rest and during exercise using 10 μC of each isotope

Preliminary experiments and calculations

Some preliminary experiments were necessary before the quantities of substances utilized or produced at rest and during exercise could be calculated

Partition of blood flow between skin and deep tissue Forearm and hand blood flow were measured in 10 healthy male subjects with venous occlusion plethysmography before and after iontoforesis of adrenaline The iontoforesis was carried out according to Cooper Edholm and Mottram (1955) who have shown that skin circulation is almost completely arrested after this treatment The arm was placed in a solution of adrenaline (1 2 000 free base) and a current of 10—15 mA was applied during 20—25 min In none of the experiments did the pulse rate or forearm blood flow increase, indicating that there was no significant absorption of adrenaline to forearm muscle or the systemic circulation The completeness of the block to the skin circulation was tested in two subjects J^{125} labelled antipyrine (10 μC in 0 1 ml saline Radiochemical Centre Amersham was injected intracutaneously into forearm skin in one subject and subcutaneously in the other In both subjects 0 1 ml of J^{131} labelled antipyrine was also injected into the flexor muscles of the forearm The clearance of the isotopes was followed with a scintillation detector placed over the injection sites and connected to a spectrometer and two ratemeters (Packard Instruments The removal of indicator from skin and subcutaneous tissue was completely stopped after the iontoforesis whereas removal of indicator from muscle was practically uninfluenced

Resting blood flow measured by venous occlusion plethysmography fell from 53 3 ± 18 9 ml min before iontoforesis to 26 8 ± 14 2 ml min after, which is a

highly significant decrease (49.9 ± 17.4 per cent). Thus in the average case under these experimental conditions approximately 50 per cent of the forearm and hand blood flow is distributed to deep forearm tissue.

Skin blood flow during exercise. J^{1} and J^{131} labelled antipyrine (10 μC in 0.1 ml) were injected with a fine needle intracutaneously into forearm skin and into forearm flexor muscle respectively. The removal of the indicators at rest and during exercise was followed with a scintillation detector placed over the injection sites as described above.

The rate of clearance from the intracutaneous depot appeared unchanged at rest and during exercise (Fig. 22) while a sharp increase was recorded in the clearance rate for the muscle indicator at the start of exercise. The results thus indicate that the forearm skin circulation is unchanged during local exercise of this type.

Forearm blood flow with elevated arm. The effect on forearm and hand blood flow at rest of raising the arm from the horizontal to the vertical position in a supine subject was investigated by plethysmographic measurements. With the arm horizontal the mean blood flow for 10 subjects was 51.5 ± 15.8 ml/min. The arm was then elevated vertically and ca. 5 min later the measurement was repeated. Blood flow was then 29.0 ± 19.1 ml/min indicating a highly significant decrease of 43.8 ± 6.7 per cent.

Calculations. The uptake to or release from the arm of the substances measured at rest (Q_o) was calculated from the following equation on the assumption that the blood flow to the hand and forearm skin equals the flow to forearm deep tissue (see above).

$$Q_o = 0.5\,F_o\,(a_o - dv_o) + 0.5\,F_o\,(a_o - sv_o) \tag{1}$$

where F_o is blood flow at rest and a, dv and sv are the arterial, deep and superficial venous concentrations of the metabolite concerned. The subscript o refers to quantities measured at rest.

During exercise skin blood flow and hence presumably skin oxygen uptake are unchanged, the increased blood flow probably going almost exclusively to muscle. The uptake or release of substances during exercise (Q) may then be calculated from

$$Q = (F - 0.5\,F_o)\,(a - dv) + 0.5\,F_o\,(a - sv_o) \tag{2}$$

The change from rest to exercise (ΔQ) is given by

$$\Delta Q = Q - Q_o \tag{3}$$

The calculation of differences in the uptake or production of substances during exercise with the arm horizontal and vertical was based on equation (2). Total blood flow was found to decrease on an average 44 per cent and this gravitational effect has been assumed to be the same for muscle as for skin.

circulation (Reller, Sheridan and Aust 1964) Skin blood flow with the arm vertical would then be 0 22 F_0 The difference due to the change in arm position in the forearm muscle uptake or production of substances was then calculated from

$$Q_h - Q_e = (F_h - 0\ 5\ F_o)(a_h - dv_h) - (F_e - 0\ 22\ F_o)(a_e - dv_e) \qquad (4)$$

where the subscripts h and e indicate properties measured with the arm horizontal and elevated respectively

Mechanical efficiency (ME) was computed according to the formula

$$ME = \frac{\text{mechanical work performed (kpm/min) } 100}{\text{(total — basal kcal prod /min) } 427}$$

Both aerobic and anaerobic metabolism were included in the calculations The changes from rest to exercise in oxygen uptake and lactate production were calculated as described above The caloric coefficient for oxygen was assumed to be 4 9 kcal/l Conversion of 1 glucosyl unit of glycogen into 2 molecules of lactate is associated with a free energy change of —57 kcal/mole glucosyl (Burton and Krebs 1953) and this value was used in the calculations

Comments The concentrations measured in deep and superficial venous blood are mainly representative of the deep forearm structures mostly muscle, and of skin and subcutaneous tissue The present measurements of forearm blood flow on the other hand give single values which do not distinguish between flow to skin and deep tissue either at rest or during exercise The presented calculations make some allowance for the variations in metabolism in the different tissues In studies on forearm metabolism at rest with arterial occlusion at the wrist, the expression $Q_0 = F_0(a_0 - dv_0)$ has been applied (Andres Cader and Zierler 1956, Baltzan *et al* 1962) This expression differs from (1) by the amount 0 5 $F_0(sv_0 - dv_0)$ Since the hand circulation could not be excluded during exercise in the present study this formula was not readily applicable moreover it would have given higher values for oxygen uptake both at rest and during exercise in comparison with those calculated from (1) and (2)

The reduction of skin blood flow after iontoforesis shows considerable inter individual variations The generalization that 50 per cent of the total flow is distributed to superficial tissue under these experimental conditions may certainly lead to errors in the individual case Fortunately this value is relatively unimportant in determining Q during exercise If for example F_0 is assumed to be 50 ml/min F is 400 ml/min $(a_T - sv_0)$ O is 40 ml/l and (a—dv) O_2 is 120 ml/l then a variation in the distribution of F_0 to the skin from 50 to 25 per cent will increase the estimated Q_O value by not more than 2 2 per cent Moreover this error is reduced when calculating ΔQ

TABLE 8 Various data obtained at rest in thirty subjects S oxygen saturation Hb haemoglobin concentration The subscripts a dv and sv indicate that the determinations were made in arterial deep or superficial venous blood The probability that the difference between venous and arterial concentrations was caused by random factors is indicated

	Mean ± SD		Mean ± SD	
Forearm volume ml	1150 ± 165	S per cent	97 4	± 1.5
Hand volume ml	432 ± 59	S_d per cent	43 4	± 13 0
Forearm circ max cm	27 9 ± 1 2	S_{sv} per cent	68 3	± 11 1
Wrist circ cm	17 7 ± 0 9	(a—dv) O_2 ml/l	101 6	± 23 3
Forearm length cm	27 9 ± 1 2	(a—sv) O ml/l	55 4	± 21 2
Skin fold thickness mm	4.2 ± 1 0	Lactate mmoles/l	0 68	± 0 19
Isometric strength kp	69 0 ± 8 8	$Lactate_{d-}$ mmoles/l	0 13*	± 0 15
Hb_a g/100 ml	13 80 ± 1 21	$Lactate_{sv-a}$ mmoles/l	0.24***	± 0.22

The calculation of changes in Q of forearm muscle during exercise with different arm positions is also subject to considerable individual errors Since only differences were computed these errors will however largely cancel out and will probably be of little importance for the means The assumption that the decrease in flow when the forearm was elevated has the same relative effect on skin and muscle blood flow is supported by results from animal experiments Limb tissue blood flow has been recorded in dogs under varying perfusion conditions by Reller Sheridan and Aust (1964) Skin and muscle circulation appeared to react identically to lowered perfusion pressures

Results

A Series 1, 2 and 3

Rest (Table 8—11)

Deep venous *oxygen saturation* was always lower than superficial venous oxygen saturation ($p < 0\,001$) It was not significantly correlated to any of the other variables recorded at rest (see Table 8) Oxygen saturation in superficial venous blood was probably significantly correlated to blood flow (0 40*) and was negatively related to arterial lactate concentration (—0 40*) Forearm *blood flow* was 60 0 ± 15 3 ml/min and calculated *oxygen uptake* 4 7 ± 1 3 ml/min The corresponding values for muscle tissue calculated for an estimated muscle fraction of 0 6 of the forearm volume (Abramson and Ferris 1940 Cooper Edholm and Mottram 1955) were 4 3 ± 1 0 ml/min 100 ml and 0 4 ± 0 1 ml/min 100 ml Blood flow at rest showed the highest primary cor

TABLE 9 Blood flow of forearm and hand at rest (F_o ml/min) in relation to skin fold thickness (mm) total forearm volume (ml) and circumference of the wrist (cm) The regression coefficients (b) and the significance of b are given for the equation which includes all the independent variables listed The residual SD and the corresponding coefficient of variation are given for each added variable The multiple correlation coefficient is indicated M ± SD for F_o = 60 0 ± 15 3 ml/min coefficient of variation 25 5 per cent

Independent variable	b	b/ε_b	Residual SD ml/min	Residual SD coeff of var	R
Skin fold thickness	9 20	4 23***	13 2	22 0	0 74
Total forearm volume	0 053	3 78**	11 6	19 3	
Wrist circumference	—7 45	—2 18*	10 9	18 2	

relation coefficient when related to skin fold thickness (0 53**) Age (0 49**), total forearm and hand volume (0 46**) and hand volume alone (0 50**) were also significantly correlated to blood flow at rest After elimination of the effect of skin fold thickness the highest partial correlation coefficient wa shown by total forearm and hand volume (0 50**) while the correlations to age and hand volume were no longer significant When the statistical effect of both *skinfold thickness and total volume was eliminated* (Table 9), a negative correlation remained to the circumference of the wrist (r = —0 39*)

Lactate concentrations were higher in superficial than in deep venous blood ($p < 0.01$) and both (v—a) differences were highly significantly different from zero No significant correlation was found to oxygen uptake or (a—v) oxygen difference The calculated forearm production of lactate was 11 ± 10 μmoles/min and the value for muscle tissue 0 6 ± 0 7 μmoles/min 100 ml

Lactate concentrations were measured in all eries in blood samples obtained immediately before the start of each exercise period Neither arterial nor (dv—a) concentration differences for lactate were significantly different at the start of the second or third exercise For practical reasons, blood flow and deep venous oxygen saturation at rest were usually determined only before the first exercise period Repeated measurements were however made in 10 of the subjects After rest periods of 35—45 min both blood flow and deep venous oxygen saturation were found not to deviate significantly from the corresponding values obtained before the first exercise period (mean differences blood flow ± 1 1 ± 4 0 ml/min $p > 0.3$, deep venous oxygen saturation + 1 2 ± 7 5 per cent $p > 0.3$)

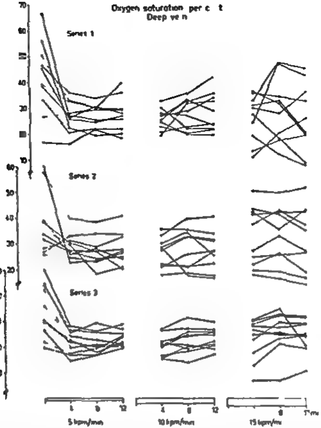

Fig 23 Oxygen saturation in blood samples from deep forearm veins at rest and after 4 8 and 12 min exercise at 5 10 and 15 kpm/min in Series 1 2 and 3 Resting values were obtained before the first period of exercise The order of exercise for the 5 and 10 kpm/min periods was randomized and the 15 kpm/min exercise was performed last Values obtained at rest and after 4 min exercise are joined by solid lines for the first exercise period and by broken lines for the second and third

Exercise

Venous oxygen saturation (Fig 23 24 Table 10) The deep venous oxygen saturation decreased at the transition from rest to exercise ($p < 0.01—0.001$) This new level of oxygen saturation was then maintained approximately constant and no differences were found between the three series Deep venous oxygen saturation was not significantly correlated to either work intensity or duration of work In all series the dispersion of deep venous oxygen saturation was greater at 15 kpm/min exercise than at 5 and 10 kpm/min The difference in oxygen saturation between values obtained at 10 kpm/min and 15 kpm/min was significantly correlated to simultaneous changes in blood flow Fig 24

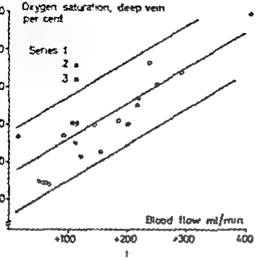

Fig 24 The regression of changes in deep venous oxygen saturation between exercise at 10 and 15 kpm/min (12 min values) on simultaneous changes in blood flow in Series 1 2 and 3 The regression line ± 2 SD ($y = 0\ 098x - 13\ 9$ SD = 5 7 r = 0 84***) is shown

TABLE 10 M ± SD for Series 1 2 and 3 for oxygen saturation of deep venous blood (S_d) $(a-dv)\ O_2$, deep venous arterial concentration difference (dv—a) and arterial and 15 kpm/min.

	Series	Rest	5 kpm/min 4 min	8 min	12 min
S_{dv}	1	41 8 ±14 3	26 8 ± 6 0	26 8 ± 4 7	28 6 ± 7 7
per cent	2	37 8 ±12 1	29 0 ± 4 9	27 2 ± 5 8	27 7 ± 6 3
	3	44 5 ±10 5	31 6 ± 4 5	31 6 ± 4 2	33 1 ± 3 1
Hb_a	1	13 68 ± 1 26	13 41 ± 1 22	13 31 ± 1 16	13 04 ± 1 02
g/100 ml	2	13 26 ± 0 65	13 02 ± 0 72	12 88 ± 0 82	12 66 ± 0 85
	3	13 20 ± 1 11	13 10 ± 1 30	12 88 ± 1 37	12 89 ± 1 72
(a—dv) O	1	101 3 ±26 4	127 0 ±15 0	126 2 ±13 8	122 2 ±13 7
ml/l	2	107 9 ±23 9	125 9 ±11 0	129 0 ±11 0	128 9 ±11 6
	3	97 9 ±19 8	121 1 ±12 1	114 9 ±23 8	119 3 ±11 2
$Lactate_d$ —	1	0 15 ± 0 20	0 55 ± 0 60	0 50 ± 0 51	0 39 ± 0 34
mmoles l	2	0 10 ± 0 11	0 51 ± 0 60	0 50 ± 0 52	0 33 ± 0 36
	3	0 13 ± 0 11	0 77 ± 0 54	0 61 ± 0 48	0 41 ± 0 45
$Lactate_a$	1	0 69 ± 0 22	0 81 ± 0 26	0 87 ± 0 28	0 74 ± 0 17
mmoles l	2	0 66 ± 0 24	0 78 ± 0 31	0 79 ± 0 27	0 77 ± 0 22
	3	0 65 ± 0 21	0 69 ± 0 19	0 70 ± 0 15	0 70 ± 0 16
Heart rate	1	57 7 ± 7 6	64 3 ±12 1	64 4 ±11 0	68 0 ±13 0
beats/min	2	57 6 ±12 8	60 9 ± 9 8	60 0 ±10 1	62 5 ±12 8
	3	59 3 ± 8 6	62.2 ± 8 4	62 1 ± 9 5	63 6 ±10 3

illustrates this relationship for values obtained after 12 min exercise Calculations with 4 and 8 min values showed similar results No significant differences were found between Series 1 2 and 3 in this respect The corresponding relationships for venous oxygen saturation and blood flow values obtained at 5 and 10 kpm/min were not significant

In Series 1 but not in the other series there was a significant correlation between deep venous oxygen saturation after 12 min exercise and T_1 (r = —0 53**) and T_6 (r = —0 51 **)

The deep venous oxygen saturation was always lower than the values obtained simultaneously for superficial venous blood (53 2 ± 10 6 per cent sv—dv 20 8*** ± 12 2) These values were calculated from observations in 25 subjects on 60 occasions during exercise in Series 1 2 and 3

arterial haemoglobin concentration (Hb) arterial—deep venous oxygen difference concentrations (a) for lactate measured at rest and after 4 8 and 12 min exercise at 5 10

10 kpm/min			15 kpm/min		
4 min	8 min	12 min	4 min	8 min	12 min
25 8 ± 4 2	27 4 ± 5 8	28 4 ± 7 3	26 1 ± 9 0	28 8 ±12 1	25 9 ±13 0
26 5 ± 5 8	27 9 ± 8 0	27 2 ± 7 4	32 6 ±11 6	33 1 ±11 1	31 3 ±12 6
30 5 ± 4 1	31 3 ± 7 0	33 2 ± 3 9	32 9 ± 8 0	35 7 ± 8 0	34 5 ± 6 4
13 54 ± 1 22	13 32 ± 1 28	13 18 ± 1 26	13 10 ± 1 16	13 07 ± 1 32	12 95 ± 1 23
13 24 ± 0 87	13 17 ± 0 72	12 96 ± 0 76	12 77 ± 0 85	12 81 ± 0 93	12 65 ± 0 84
13 07 ± 1 09	12 95 ± 1 24	13 13 ± 1 49	12 63 ± 1 09	12 49 ± 1 28	12 57 ± 1 27
130 6 ± 14 0	125 1 ± 12 8	122 2 ± 14 8	124 0 ± 16 7	127 8 ± 26 0	125 4 ± 29 5
131 8 ± 13 5	126 8 ± 16 9	128 6 ± 15 3	121 0 ± 24 0	120 1 ± 23 5	123 9 ± 25 3
122 9 ± 11 8	122 3 ± 15 2	118 1 ± 10 4	121 0 ± 14 3	116 6 ± 12 4	118 4 ± 11 4
1 41 ± 0 70	1 45 ± 1 00	1 28 ± 0 89	2 91 ± 1 33	3 02 ± 1 41	2 48 ± 1 55
1 93 ± 0 87	1 82 ± 0 91	1 81 ± 1 39	2 45 ± 0 88	1 93 ± 0 96	1 56 ± 1 08
1 72 ± 1 80	1 64 ± 1 46	1 26 ± 1 23	2 55 ± 1 16	1 77 ± 0 86	1 33 ± 0 81
0 87 ± 0 29	0 91 ± 0 33	0 86 ± 0 35	0 98 ± 0 33	1 23 ± 0 40	1 43 ± 0 56
0 83 ± 0 13	0 88 ± 0 12	0 89 ± 0 17	0 97 ± 0 17	1 06 ± 0 41	1 06 ± 0 22
0 85 ± 0 22	0 92 ± 0 28	0 97 ± 0 22	0 94 ± 0 23	0 98 ± 0 25	1 11 ± 0 47
68 2 ±13 7	67 9 ±13 4	69 2 ±12 9	77 0 ±16 5	84 0 ±18 6	86 6 ±19 2
64 7 ±14 3	68 2 ±15 6	68 4 ±15 3	73 4 ±17 6	76 6 ±19 0	82 4 ±22 3
64 8 ± 8 0	67 5 ±10 8	64 7 ± 9 3	69 0 ±12 5	72 6 ±13 8	71 4 ±12 2

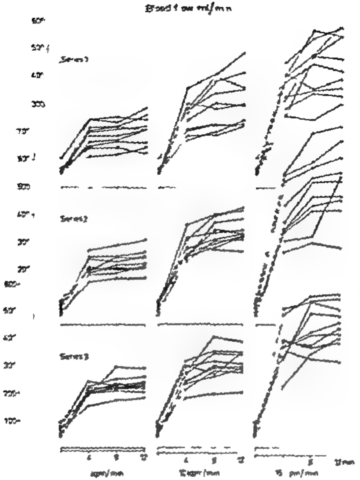

Fig. 2. Blood flow at rest and after 4, 8 and 12 min exercise at 5, 10 and 15 kpm/min in Series 1, 2 and 3. Symbols as in Fig. 23

Deep venous oxygen saturation as dependent variable (10 kpm/min). Deep venous oxygen saturation after 12 min exercise was negatively correlated to T_e ($r = -0.41^*$) and to the (dv—a) lactate difference ($r = -0.39^*$). After elimination of the effect of T_e in the calculations, the correlation to deep venous oxygen saturation at rest was probably significant ($r = 0.38^*$). None of the variables describing the dimensions of the forearm were probably significantly correlated. Similar correlations to T values were not found for deep venous oxygen saturation obtained after 4 or 8 min exercise.

Haemoglobin concentration (Table 10). The haemoglobin concentration decreased by $0.22^{**} \pm 0.39$ g/100 ml during the course of exercise, probably because of dilution with the dye solution. Arterial haemoglobin concentration did not vary significantly between the different series.

(a—dv) oxygen difference (Table 10) The changes in (a—dv) oxygen difference corresponded closely to the pattern to be expected from the simultaneous variations in deep venous oxygen saturation However, while the deep venous oxygen saturation remained unchanged during work a small decrease was noted in the (a—dv) oxygen difference between 4 and 12 min at 10 kpm/min ($p < 0.05$) This may have been an effect of the lowered haemoglobin concentration caused by the infused dextran solution

Blood flow (Fig 25, 26, Table 11 12) During exercise at 5 kpm/min blood flow reached a fairly steady level after 4 min with only a small further increase during the following 8 min (Series 2 $p < 0.01$ Series 1 and 3 $p < 0.05$) At 10 and 15 kpm/min the rise between 4 and 12 min was usually more pronounced especially at 15 kpm/min in Series 2 ($p < 0.001$) In Series 3 however, only a small increase was found between 4 and 12 min ($p < 0.05$)

The increase in blood flow during exercise was linearly related to work intensity within the range tested Blood flow per 100 ml forearm tissue showed no closer correlation to work intensity, nor was work intensity per 100 ml tissue better correlated to blood flow The slope of the regression of blood flow after 12 min exercise on work intensity was probably significantly steeper in Series 2 than in Series 3 otherwise no differences were found between the series The regression of blood flow on work intensity combined with the different times recorded for contraction and relaxation (T_1—T_6) as a second independent variable was calculated for Series 1, 2 and 3 In Series 1 the addition of T_1—T_6 respectively to work intensity as independent variables decreased the residual standard deviation (Table 13) The regression coefficients were negative for all T values except T_4 The largest reduction in the residual standard deviation was found when T_4 and T_5 were included in the calculations The combination of T values and work intensity per 100 ml forearm tissue or the use of blood flow per unit tissue as dependent variable did not improve these relationships Similar relationships were not found in Series 2 and 3

Blood flow as dependent variable (10 kpm/min) The increase in blood flow from rest to exercise was preferred in the statistical calculations since it showed similar but almost always slightly better correlations than total blood flow The change in blood flow after 4 min exercise was correlated most closely to the maximal circumference of the forearm (CA $r = 0.52^{**}$) of the variables describing the dimensions of the arm With the effect of CA eliminated the highest partial correlation coefficients were shown by T_1 (-0.42^{*}) and T_5 (-0.39^{*}) The multiple correlation coefficient for CA and T_1 combined was 0.63^{***} The blood flow change after 8 min exercise showed similar correlations

TABLE 11. M ± SD for Series 1, 2 and 3 for blood flow (F), oxygen uptake (Q_{O_2}), production, holding (T_2) and relaxation (T_3) measured at 4, 8 and 12 min during exercise

	Series	Rest	5 kpm/min 4 min	8 min	12 min
F	1	62 ± 17	182 ± 44	193 ± 43	205 ± 52
ml/min	2	63 ± 17	210 ± 35	213 ± 45	230 ± 2
	3	67 ± 17	205 ± 29	226 ± 32	239 ± 40
Q_{O_2}	1	46 ± 12	214 ± 60	218 ± 57	223 ± 69
ml/min	2	51 ± 29	234 ± 41	247 ± 38	270 ± 36
	3	51 ± 10	227 ± 34	252 ± 36	253 ± 38
Q_{lact}	1	12 ± 13	104 ± 108	77 ± 80	76 ± 67
µmoles/min	2	5 ± 8	95 ± 97	98 ± 106	68 ± 65
	3	12 ± 7	145 ± 103	124 ± 95	99 ± 85
MF	1		158 ± 63	150 ± 57	147 ± 65
per cent	2		135 ± 34	117 ± 22	119 ± 17
	3		132 ± 38	120 ± 29	120 ± 25
T_1	1		235 ± 55	227 ± 55	229 ± 57
msec	2		176 ± 79	223 ± 99	244 ± 53
	3		123 ± 34	138 ± 50	135 ± 44
T_2	1		163 ± 40	155 ± 34	164 ± 41
msec	2		141 ± 53	151 ± 38	163 ± 71
	3		126 ± 23	126 ± 10	113 ± 15
T_3	1		235 ± 54	260 ± 76	27[illegible] ± 77
msec	2		261 ± 106	303 ± 182	375 ± 238
	3		167 ± 65	162 ± 70	179 ± 81

For the change in blood flow after 12 min exercise the highest primary correlation coefficients were found for T_3 (0.47** and T_2 (−0.47**). The other T-values showed similar negative correlations (T_1 −0.29, T_2 −0.45*, T −0.50*, T_3 −0.43*). The dimensions of the forearm and hand were not significantly correlated to the change in blood flow during exercise in contrast to the findings for blood flow measured at 4 and 8 min. When partial correlation coefficients were calculated after eliminating the effect of T, the correlation to CA was probably significant (0.37*). The variables T and CA combined gave a multiple correlation coefficient of 0.57**.

The increase in blood flow from 4 to 12 min during exercise was 57.1 ± 32.1 ml/min. This increase was correlated to the change in heart rate from rest to 12 min exercise (r = 0.48**).

of lactate ($Q_{lactate}$) mechanical efficiency (ME) and time of contraction (T_1) isometric at 5 10 and 15 kpm/min and at rest where applicable

10 kpm/min			15 kpm/min		
4 min	8 min	12 min	4 min	8 min	12 min
253 ± 60	298 ± 81	315 ± 91	353 ± 68	432 ± 102	456 ± 108
276 ± 42	332 ± 47	345 ± 49	403 ± 104	486 ± 124	524 ± 129
256 ± 42	301 ± 58	306 ± 52	374 ± 80	436 ± 87	449 ± 77
30 4 ± 8 1	34 9 ± 10 3	35 9 ± 10 9	41 6 ± 10 9	50 1 ± 15 2	53 2 ± 12 6
33 9 ± 5 4	39 1 ± 2 9	41 9 ± 5 6	45 3 ± 7 4	48 8 ± 18 0	60 5 ± 10 0
29 0 ± 4 3	34 7 ± 9 6	33 8 ± 5 5	47 8 ± 9 9	48 7 ± 10 5	50 7 ± 8 1
303 ± 137	399 ± 308	366 ± 269	965 ± 600	1134 ± 452	967 ± 465
468 ± 198	530 ± 237	549 ± 403	887 ± 367	851 ± 399	690 ± 390
381 ± 397	489 ± 498	376 ± 413	866 ± 427	671 ± 278	531 ± 259
18 9 ± 4 6	16 7 ± 6 5	15 9 ± 5 3	18 4 ± 5 9	14 9 ± 3 8	14 0 ± 3 6
15 6 ± 2 5	12 8 ± 1 3	11 1 ± 4 0	16 3 ± 2 7	12 9 ± 4 9	12 4 ± 2 2
19 5 ± 5 1	16 8 ± 6 1	16 5 ± 5 0	17 9 ± 4 4	15 7 ± 3 53	15 1 ± 2 4
21 7 ± 5 8	23 7 ± 6 7	22 7 ± 6 ?	24 1 ± 6 9	26 4 ± 9 3	23 3 ± 4 5
18 6 ± 5 1	19 1 ± 5 4	17 0 ± 4 0	14 9 ± 5 3	14 9 ± 3 5	15 7 ± 3 9
19 8 ± 6 6	19 1 ± 6 8	19 5 ± 5 3	26 6 ± 10 0	24 4 ± 8 2	24 0 ± 6 0
13 1 ± 2 0	13 7 ± 3 4	12 8 ± 3 2	13 8 ± 3 0	14 5 ± 3 6	12 5 ± 2 8
14 7 ± 2 7	13 7 ± 4 5	13 0 ± 2 7	11 4 ± 3 5	10 5 ± 3 7	10 6 ± 3 4
13 5 ± 1 6	13 2 ± 2 3	13 0 ± 1 1	13 3 ± 3 4	12 0 ± 2 7	12 1 ± 2 1
19 9 ± 7 7	22 8 ± 6 5	22 5 ± 9 2	22 3 ± 2 6	20 7 ± 6 5	19 6 ± 8 0
21 3 ± 8 6	22 0 ± 9 9	22 0 ± 11 7	15 9 ± 7 4	16 0 ± 7 2	17 3 ± 7 3
23 2 ± 9 2	21 3 ± 9 4	23 8 ± 11 1	27 2 ± 10 5	24 5 ± 10 5	24 7 ± 10 2

Oxygen uptake (Fig 26 27 Table 11 12) The time course for oxygen uptake during exercise followed approximately that for blood flow At 5 kpm/min a steady level of oxygen uptake was reached after 4 min exercise Only in Series 2 was a significant further increase observed between 4 and 12 min exercise At 10 and 15 kpm/min the rise in oxygen uptake between 4 and 12 min was larger and significant in all series

The oxygen uptake during exercise increased linearly with work intensity The increased oxygen uptake was accomplished almost exclusively by the rise in blood flow since deep venous oxygen saturation remained constant on the average Regression calculations using oxygen uptake per 100 ml tissue or work intensity per unit tissue did not give closer correlations The slope of the regression of oxygen uptake on work intensity was significantly steeper in

TABLE 12 The regression of the change from rest to after 12 min exercise for blood flow (ΔF ml/min) oxygen uptake (ΔQ_{O_2} ml/min) and log lactate production ($\Delta Q_{lactate}$ µmoles/min) on work intensity (W kpm/min) and work intensity per 100 ml forearm tissue (rW kpm/min/100 ml) The regression of log deep venous arterial lactate concentration difference ($Lactate_{dv-a}$ mmoles/l) on rW is also given For the exponential functions the SD is given in log units The significance of the regression coefficients (b/s_b) was $p < 0.001$ in all cases

Series	Dependent variable	Independent variable	Regression equation	± SD	b/s_b
1	ΔF	W	$y = 24.79x + 17.2$	85.9	6.45
2	ΔF	W	$y = 29.51x + 8.2$	79.4	8.31
3	ΔF	W	$y = 21.95x + 42.3$	55.1	8.90
1	ΔQ_{O_2}	W	$y = 3.032x + 2.40$	10.0	6.77
2	ΔQ_{O_2}	W	$y = 3.348x + 4.55$	6.6	11.31
3	ΔQ_{O_2}	W	$y = 2.546x + 6.04$	6.2	9.19
1	log $\Delta Q_{lactate}$	W	$y = 0.3627x + 1.506$	1.746	4.65
2	*log $\Delta Q_{lactate}$	W	$y = 0.3176x + 2.049$	1.381	5.14
3	*log $\Delta Q_{lactate}$	W	$y = 0.2124x + 3.035$	0.906	5.24
1	*log $\Delta Q_{lactate}$	rW	$y = 3.668x + 1.923$	1.757	4.57
2	log $\Delta Q_{lactate}$	rW	$y = 3.477x + 2.089$	1.345	5.42
3	*log $\Delta Q_{lactate}$	rW	$y = 2.531x + 3.030$	0.852	5.89
1	log $Lactate_{dv-a}$	rW	$y = 2.307x - 2.280$	0.965	5.24
2	log $Lactate_{dv-a}$	rW	$y = 2.449x - 2.676$	1.324	3.88
3	log $Lactate_{dv-a}$	rW	$y = 1.770x - 1.967$	0.825	4.24

Series 2 than in Series 3 When both work intensity and T values were used simultaneously as independent variables negative regression coefficients (b) were found for T_3 ($b = -2.93^{**}$) and T_5 ($b = -2.17^{*}$) The partial correlation coefficients for the T values in these calculations were always lower than those obtained with blood flow as dependant variable indicating that the correlation between the T values and oxygen uptake was caused by the correlation between blood flow and T values

Lactate (Fig 28 29 Table 10—12) The (dv—a) lactate difference was highest after 4 or 8 min exercise and had usually decreased at 12 min This reduction was accompanied by the simultaneous increase in blood flow but lactate production especially at 10 and 15 kpm/min nevertheless usually *decreased at the end of exercise (Series 3 15 kpm/min $p < 0.01$)*

During exercise both the (dv—a) lactate concentration difference and the total lactate production showed an approximately exponential increase with

TABLE 13 The regression of the blood flow increase from rest to after 12 min exercise in Series 1 on work intensity (W kpm/min) in combination with the time taken to move the handles the required path of contraction (T_1 csec) the duration of isometric contraction (T_2 csec) and the duration of relaxation (T_3 csec) T_4 is the time from the end of one relaxation till the start of the following contraction T_5 is the sum of T_1 T_2 and T and T_6 is the sum of T_1 and T_2

Independent variables		Regression equation	Residual SD	b/ϵ_b
W		$y = 24.79x + 17.23$	85.9	6.45***
W	x_1	$y = 25.00x_1 - 6.93x_2 + 158.1$	80.6	6.94**
T_1	x_2			−2.21*
W	x_1	$y = 21.90x_1 - 7.45x + 149.5$	83.6	5.29***
T_2	x			−1.63
W	x_1	$y = 19.54x - 5.90x_2 + 208.6$	70.9	5.63***
T_3	x_3			−3.75**
W	x_1	$y = 20.15x_1 + 3.73x - 84.6$	70.4	5.97***
T_4	x			3.85**
W	x_1	$y = 20.13x - 3.74x + 289.7$	70.4	5.96*
T_5	x			−3.86**
W	x	$y = 22.57x - 6.26x_2 + 270.0$	76.9	6.40***
T	x_6			−2.83**

rising work intensity Log lactate production gave higher correlation co efficients than log lactate (dv—a) difference when related to work intensity Closer correlations still were obtained with the regression of log lactate pro duction on work intensity per unit forearm tissue The slope of this regression for log lactate production after 8 min exercise was probably significantly steeper for Series 1 than for both the other series Otherwise no significant differences were found between the series

Lactate (dv—a) difference as dependent variable (10 kpm/min) The loga rithm of the (dv—a) lactate difference after 4 min exercise was negatively correlated to the circumference of the arm at the wrist (CW r = —0 63**) and maximal circumference of the forearm (r = —0 46**) and also to total forearm and hand volume (r = —0 56***) hand volume (r = —0 59**) and forearm volume (r = —0 52**) Blood flow at rest was also negatively cor related (r = —0 43*) to the lactate (dv—a) difference

After 8 and 12 min exercise log (dv—a) lactate difference showed similar correlations to the dimensions of the forearm Values obtained at 12 min were also correlated to (a—dv) oxygen difference (r = 0 53**) After elimination of the effect of CW significant partial correlation coefficients were found for

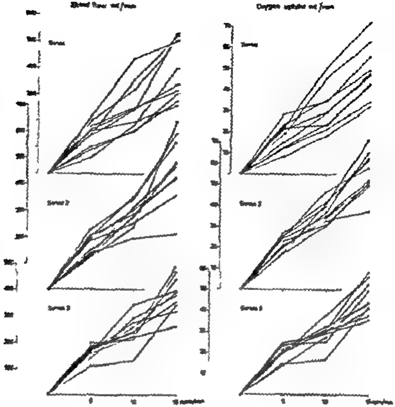

Fig. 26. The increase in blood flow and in oxygen uptake from rest to at 12 min exercise as functions of work intensity in Series 1, 2 and 3. The regression equations are given in Table 12.

the change from rest to exercise in oxygen uptake ($r = 0.54^{**}$) and for (a—dv) oxygen difference ($r = 0.47^{**}$). The correlations to blood flow and T values were not significant.

The decrease in (dv—a) lactate difference between 8 and 12 min during exercise was probably significant ($0.20^{*} \pm 0.53$ mmoles/l) and was negatively correlated to the (a—dv) oxygen difference at 12 min exercise ($r = -0.43^{*}$).

Mechanical efficiency (Table 11). The mechanical efficiency usually decreased during exercise. The increase in oxygen uptake was not balanced by an equivalently large reduction in lactate production and there was a decrease in the calculated mechanical efficiency, especially at the higher work intensities (Series 1, 2 and 3, 10 and 15 kpm/min, $p < 0.01$). At 5 kpm/min there was no significant change in mechanical efficiency between 4 and 12 min exercise.

The highest mechanical efficiency was usually found for exercise at 10 kpm/min. In Series 2, however, slightly, but not significantly higher values were found for 15 kpm/min. No significant differences were observed between Series

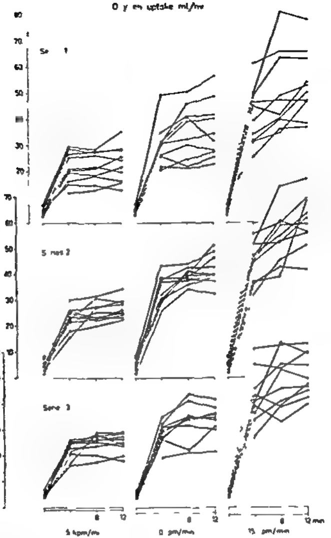

Fig 27 Oxygen uptake at rest and after 4 8 and 12 min exercise at 5 10 and 15 kpm/min in Series 1 II and 3 Symbols as in Fig 23

1 2 and 3 Mechanical efficiency was not significantly correlated to any of the variables recorded at rest

T values (Table 11) In Series 1 the T values did not change significantly during the exercise periods However both T_2 and T_3 were significantly longer at 12 min of 5 kpm/min exercise than after the same time at 10 and 15 kpm/min while T_1 remained unchanged Blood flow and oxygen uptake were found to be significantly related to some of the T values in Series 1 (see above)

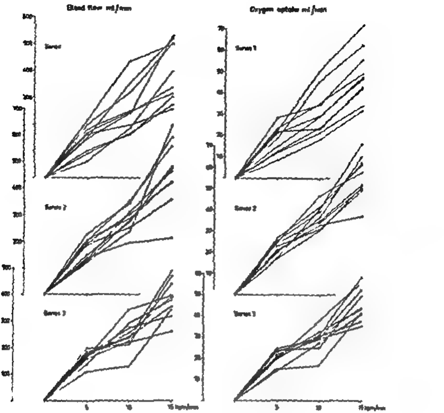

Fig 26 The increase in blood flow and in oxygen uptake from rest to at 12 min exercise as functions of work intensity in Series 1 2 and 3 The regression equations are given in Table 12

the change from rest to exercise in oxygen uptake ($r = 0.54^{**}$) and for (a—dv) oxygen difference ($r = 0.47^{**}$) The correlations to blood flow and Γ values were not significant

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The highest mechanical efficiency was usually found for exercise at 10 kpm/min In Series 2 however slightly but not significantly higher values were found for 15 kpm/min No significant differences were observed between Series

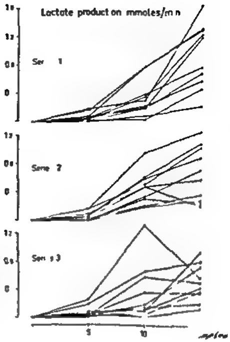


Fig 29 The change in lactate production from rest to at 12 min exercise as a function of work intensity in Series 1 2 and 3 The regression equations are given in Table 12

Heart rate (Table 10) During the course of exercise the heart rate increased successively but rather moderately The rise between 1 and 2 was significant only during 15 kpm/min exercise in Series 1 and 2

The heart rate increased approximately linearly with the work in Series 1 and 2 it was significantly higher at 10 than at 5 kpm/min and at 15 than at 10 kpm/min in Series 3 these two differences were not significant

Heart rate as dependent variable (10 kpm/min) The increase from rest to 4 and 8 min exercise was correlated most closely to the lactate production from rest to exercise (r = 0 49** r = 0 69*** exercise the change in heart rate was again correlated to the lactate production (r = 0 68***) as well as to the (dv—a) lactat 0 61***) Of the variables describing the dimensions of the f only hand volume showed a probably significant correlation

B Series 4

Rest (Table 14)

When the subjects in the supine position elevated the artery mean blood pressure (measured with the intra ence level) decreased 24 1*** ± 5 2 mm Hg Systoly

Table 14 Summary of some of the data obtained in Series 4 M ± SD are given for the differences in the properties measured at rest and during exercise with the arm horizontal and vertically elevated The probability that the differences were caused by random factors is indicated Symbols and units as in Tables 10 and 11

Effect of change from horizontal position to vertical on	Rest	Exercise 4 min	[illegible] min	12 min
F		—49 8*** ± 17 5	—59 2*** ± 32 2	—48 5*** ± 32 8
S_d	—11 4* ± 12 0	— 9 8*** ± 4 0	—11 8*** ± 5 0	—9 5*** ± 6 0
Q_O		—1 3 ± 2 9	0 1 ± 4 6	0 1 ± 2 2
$Lactate_{d-a}$	0 03 ± 0 17	1 30*** ± 0 92	1 57** ± 1 42	1 21** ± 0 93
$Lactate_a$	—0 11* ± 0 14	0 15* ± 0 16	0 05 ± 0 19	0 04 ± 0 27
Q_{lactat}		206* ± 207	310** ± 290	238** ± 210
Heart rate	0 ± 7	[illegible] ± 9	5 ± 12	5 ± 8

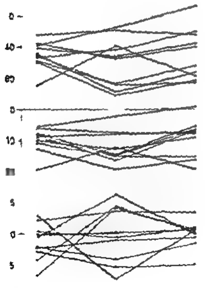


Fig 30 Differences between exercise with the forearm horizontal and vertically elevated in blood flow (F ml/min) deep venous oxygen saturation (S_{dv} per cent) oxygen uptake (Q_O ml min) and lactate production ($Q_{lactate}$ mmoles/min) Values calculated for 4 8 and 12 min exercise are given

were reduced by almost identical amounts Oxygen saturation in blood from deep forearm veins (obtained with the forearm elevated for at least 5 min) was 37 3 $\pm$ 8 0 per cent which is probably significantly lower than the corresponding value with the arm horizontal (48 7 $\pm$ 13 8 per cent) It was difficult to draw blood from the superficial veins with the arm raised However in four experiments samples were obtained and a mean decrease of 24 1 per cent in oxygen saturation was found (range 2 3—48 5 per cent)

The lactate concentration in deep venous blood did not change significantly when the forearm was elevated

Exercise (Fig 30 Table 14)

The subjects performed exercise at 10 kpm/min for 12 min both with the arm horizontal and with it vertically elevated The results are expressed as the difference between the two exercise periods Both blood flow and deep venous oxygen saturation were significantly reduced during exercise with the arm raised The difference in calculated oxygen uptake was not significantly different from zero However there was a significant rise in both (dv—a) lactate concentration difference and lactate production For none of the calculated differences were significant changes found between values obtained at 4 8 and 12 min The data obtained were not significantly correlated to the decrease in brachial artery mean pressure The increase in heart rate was not significantly higher during exercise with the arm elevated as compared to at horizontal exercise

Discussion

Characteristics of the exercise performed

The exercise performed by the subjects in Series 1—4 has been termed isotonic indicating that the muscles shorten under constant load It should be pointed out however that the present form of exercise is not made up of genuinely isotonic contractions Owing to the tension length characteristics of the different springs used in the hand ergometer the tension increases slightly as the spring is stretched Moreover when the subjects have completed the pre set path of contraction of the hand ergometer tension of the forearm muscles is unavoidably maintained for a short time in an isometric contraction In addition the recordings of the time course of contraction and relaxation do not tell whether the subjects made a further increase in tension during this isometric phase However the subjects were always carefully instructed to terminate the contraction as soon as they heard the signal from the ergometer micro switch indicating that the path of contraction was complete and it usu

ally took less than a minute for the subjects to adapt themselves to the rhythm of the exercise Neither the duration of the isometric contraction (T) nor the duration of the shortening phase plus the isometric contraction (T_c) was significantly correlated to oxygen uptake It is thus unlikely that the isometric phase constituted an important part of the exercise performed

Venous oxygen saturation

The oxygen saturation of forearm deep venous blood was found to decrease significantly at the transition from rest to exercise This is in agreement with earlier observations (Love 1955, Monod *et al* 1961, Pernow and Wahren 1962) During exercise the oxygen saturation remained approximately constant regardless of the work intensity used The values obtained agree closely with those reported by Greenwood, Robards and Twite (1965) for deep venous blood during rhythmic squeezing of a water filled rubber bulb Monod *et al* (1958 and 1961) have studied the venous oxygen content during exercise with a hand ergometer (path of contraction 40 mm frequency 30 per min) At exercise with different work intensities, produced by changing the load per contraction, deep venous oxygen content remained constant at approximately 7 5 vol per cent This finding is confirmed in the present study in Series 1, although the level of oxygen saturation is slightly lower Similar results were also obtained when work intensity was altered by changing the frequency of contraction (Series 2) or the path of contraction (Series 3)

The values for deep venous oxygen saturation showed a considerable dispersion especially at 15 kpm/min The larger dispersion at 15 than at 10 kpm/min is partly ascribable to the considerable individual variation in the blood *flow increase from 10 to 15 kpm/min viz there was a significant correlation* between this increase and simultaneous changes in deep venous oxygen saturation in Series 1 2 and 3 A small rise in blood flow was accompanied by a reduction of deep venous oxygen saturation and vice versa (Fig 24)

Notwithstanding this correlation the interindividual dispersion of deep venous oxygen saturation is fairly considerable The combined results from Series 1 2 and 3 for 10 kpm/min showed a probably significant negative correlation between deep venous oxygen saturation and the duration of contraction plus isometric holding (T_c) This indicates that a long duration of T_c and hence possibly a large mechanical obstruction to blood flow was associated with a low deep venous oxygen saturation Blood flow showed a similar negative correlation to T_c which may indicate that the oxygen uptake was uninfluenced by variations in T_c

When supine subjects changed the position of the arm from horizontal to vertically elevated the deep venous oxygen saturation showed a marked de

crease at rest (Holling and Verel 1957) Since the transmural pressure across the vessel walls was lower with the arm elevated the resistance to flow increased and blood flow decreased (Holling and Verel 1957, Abramson *et al* 1962) During exercise with the forearm elevated in the present study, the deep venous oxygen saturation was ca 20 per cent as compared to ca 30 with the arm horizontal In other words the reduced blood flow was compensated for by a higher degree of oxygen extraction from the blood which was so balanced as to maintain a constant oxygen uptake within the error of measurement This finding is thus another illustration of the relationship described in Fig 24 between changes in blood flow and deep venous oxygen saturation

Blood flow

Resting blood flow of the forearm and hand was significantly correlated to skinfold thickness and to variables describing the dimensions of the arm notably the total forearm volume The correlation to skinfold thickness suggests that subcutaneous blood flow may be a varying component of total forearm blood flow at rest Accordingly the fixed partition factor (50 per cent) used to differentiate between blood flow to superficial and deep forearm tissue in the present study may not be strictly accurate for calculations of forearm metabolism In practice however it seems to be the only possibility at present and as demonstrated above this factor is quantitatively rather unimportant for the determination of forearm metabolism during exercise

Exercise elicited a rapid increase in blood flow a fairly steady level being reached after 4 min at work intensity 5 kpm/min At 10 and 15 kpm/min a steady state had not always been attained even after 8 and 12 min exercise. The measurement of blood flow with the present technique requires a relatively steady state during the time for infusion of dye and sampling of blood (ca 1 min 15 sec) The observed changes in blood flow during this time were small in comparison to the total blood flow but they probably caused an error in the blood flow determination which although small must be added to the error of the method

The increase in blood flow was linear for the range of work intensity investigated The subjects usually stated that exercise at 15 kpm/min was heavy but none of them had to break off because of exhaustion The blood flow at 15 kpm/min calculated per 100 ml forearm tissue was 38—44 ml/min 100 ml in Series 1 2 and 3 Folkow Grimby and Thulesius (1958) found that maximal forearm blood flow after ischemic exercise was ca 53 ml/min 100 ml Thus although the experimental conditions are not fully comparable the highest work intensity in the present study seems to have been submaximal in the average case

In Series 1 the blood flow was correlated to the durations of some of the phases of contraction and relaxation T_4 and T were equally effective in reducing the residual standard deviation when included together with work intensity as independent variables in regression analysis indicating that the subjects kept very good time with the metronome The regression coefficient was positive for T_4 and negative for T, which may indicate that a rapid contraction and relaxation impedes blood flow least and that high T values are associated with prolonged mechanical obstruction during contraction and hence a low blood flow It is, however, also possible that the negative correlation between blood flow and T is due to an active engagement of the extensor muscles during exercise with short T values, since T_3 was almost as effective as T in reducing the residual standard deviation

Similar correlations were not found between blood flow and T values in Series 2 and 3 although the dispersion of T values was approximately equal in the different series The effect of the change in the frequency of contraction (Series 2) or in the path of contraction (Series 3) on the T values may have been sufficient in itself to obscure possible correlations to blood flow

The combined results for Series 1—3 at 10 kpm/min showed significant correlations between blood flow at 4 and 8 min and variables describing the dimensions of the arm whereas the correlations to the T values were not significant Thus blood flow of a large forearm was higher after 4 and 8 min than that of a small forearm At 12 min exercise however, the correlations to forearm dimensions had lost significance while those to variables describing the manner in which the exercise was performed had become significant As was the case for blood flow during exercise in Series 1, the highest correlation coefficients were found for T_4 and T It is thus apparent that at this work intensity the magnitude of forearm blood flow is independent of the forearm dimensions when the flow is near or at a steady level This is also illustrated by the fact that the standard deviation for the regression of blood flow on work intensity did not decrease for Series 1 2 and 3 when one or other of the variables was expressed in units per 100 ml forearm tissue

Oxygen uptake

Since deep venous oxygen saturation and (a—dv) oxygen difference remained approximately constant during exercise of different work intensities the rise of oxygen uptake between 5 and 15 kpm/min was almost exclusively accomplished by the increased blood flow As shown in Fig 27 and 25 the variations in oxygen uptake are very similar to those of blood flow These figures also illustrate that at both 10 and 15 kpm/min exercise blood flow and oxygen uptake usually continue to increase even after 4 and 8 min which seems a

remarkably long time for the muscles to reach a steady state level This finding may possibly imply that auxiliary muscles are activated during the later part of the exercise period

The slopes of the regressions of both blood flow and oxygen uptake on work intensity were significantly steeper in Series 2 than in Series 3 With a reservation for the unlikely possibility that the results were influenced by effects of the isometric phase during exercise this difference between the slopes indicates that when work intensity is raised by increasing the frequency of contraction there is a greater increase in blood flow and oxygen uptake than when the path of contraction is increased In the former case more energy is expended by the series coupled elastic component in muscle contraction mostly due to stretching of the tendons it seems reasonable that this type of exercise requires a greater oxygen uptake than exercise with a longer path of contraction and lower frequency

Whereas significant correlations were found between blood flow and T_3—T_f in Series 1 and T_4 and T in the combined material at 10 kpm/min oxygen uptake was correlated only to T_3 both in Series 1 ($p < 0.01$) and in the combined material ($p < 0.025$) The results indicate that variations in the other T values were balanced by simultaneous changes in deep venous oxygen saturation As mentioned above, such a relationship was in fact found for T_f blood flow and deep venous oxygen saturation These findings support the view that the relationship between blood flow and T_3 may have been due to active extensor muscle engagement since mechanical obstruction to blood flow would probably not decrease the oxygen uptake

Lactate

During exercise the (dv—a) lactate difference and lactate production showed maxima after 4 or 8 min and had usually decreased at 12 min Since blood flow and oxygen uptake increased during exercise and reached their highest values at 12 min the reduction in lactate production was probably caused by improved oxygenation of the muscles at the end of the exercise period This is in agreement with the time course for blood lactate concentrations during leg exercise (Bang 1936) and with the observations of Monod *et al* (1961) on changes in deep venous blood lactate concentration during forearm exercise The finding of an approximately exponential increase in lactate (dv—a) difference and production with work intensity also corresponds with the lactate concentration of femoral venous blood during leg exercise (Carlson and Pernow 1961 Pernow Wahren and Zetterquist 1965)

The (dv—a) lactate difference at 10 kpm/min was significantly and negatively correlated to variables describing the dimensions of the forearm it

seems that a small forearm produces more lactate than a large one, since it exercises at a higher work intensity per unit muscle tissue Exercise with small muscle groups is generally associated with higher blood lactate concentrations (Duner 1959, Pernow, Wahren and Zetterquist 1965, Bevegård, Freyschuss and Strandell 1966)

Exercise with the forearm vertically elevated was accompanied by a significant increase in lactate production compared to the results with the arm horizontal This may indicate that the oxygen uptake, although it showed no significant difference, did not in fact reach quite the same level with the arm vertical as horizontal Other possibilities are at hand The deep venous oxygen saturation was reduced in these experiments and the intracellular pO_2 of muscle tissue may also have been lower This, in turn, may have elicited an increased lactate production by a shift in the pyruvate lactate equilibrium Similar results for oxygen uptake and blood lactate have been obtained during bicycle exercise at normal and at low partial pressures of oxygen (Christensen and Forbes 1937 Astrand 1954)

Pyruvate concentrations were also measured and excess lactate values (Huckabee 1959) calculated in the present study With few exceptions the correlations for excess lactate and total lactate were almost identical (Wahren to be published)

Mechanical efficiency

Mechanical efficiency was calculated on the basis of measured oxygen uptake and lactate production Conversion of 1 mole of pyruvate into lactate is accompanied by oxidation of 1 mole of NADH irrespective of whether this equilibrium change is accomplished by changes in NADH or pyruvate concentrations (Wasserman Burton and Van Kessel 1965) Lactate values were therefore used rather than the corresponding excess lactate (Huckabee 1959) Burton and Krebs (1953) found the conversion of glycogen into lactate in muscle extracts to be associated with a free energy change of —57 kcal per mole glucosyl equivalent and this value was used in the present study It should be remembered however that if glucose is used instead of glycogen as starting material the net energy gain will be lower since ATP is required in the hexokinase reaction

The mechanical efficiency is determined mainly by the external conditions for the exercise performed In the hand ergometer used in the present study the movement of the handles was taken up by axial ball bearings and transmitted to the spring Energy loss due to friction and unrecorded external work was probably low Isometric contraction of the forearm and hand muscles is probably used however for fixation of the wrist and thumb during finger

flexion which may be one of the reasons why the present values for mechanical efficiency are lower than those given for pedalling a bicycle ergometer with the legs (Åstrand 1960 Poulsen and Asmussen 1962) or for cranking a bicycle with the arms (Bevegard, Freyschuss and Strandell 1966). The continuous circular movement on a bicycle ergometer probably gives better efficiency than a sequence of rhythmic contractions and relaxations which heightens the expenditure of energy on tension in the series-coupled elastic component within the muscle. The exercise performed in a step test which is more comparable to the present form of hand ergometer exercise also shows lower values for mechanical efficiency (Ryhming 1953) than bicycle exercise with the legs. Scherrer Monod and Soula (1960) calculated the mechanical efficiency for hand ergometer exercise from the increase in pulmonary oxygen uptake during exercise. The values given agree closely with those found in the present study.

CHAPTER VI

Circulatory and Metabolic Response to Rhythmic Isometric Exercise

Subjects

The subjects were 10 healthy male volunteers aged 23—36 (M = 26.2 years). Five were students and the other five were firemen or policemen. All subjects attended regular health controls and were in good health at the time of the investigation.

Procedure

The experiments were performed in the morning with the subjects fasting. Measurements at rest, catheterization, collection of blood samples, analyses and calculations were carried out as described in Chapter V. The subjects then performed rhythmic isometric exercise on a strain gauge dynamometer (see General Procedures). The frequency of contraction was 60 per min. Three different exercise periods, each lasting 12 min, were carried out with rest periods of 35—45 min in between. The nominal peak tension developed for

TABLE 1[illegible]. Summary of data obtained at rest and during isometric exercise at varying contraction (kp). Other symbols and units as in Tables 10 and 11.

		10 kp		
	Rest	4 min	8 min	12 min
S_d	17.6 ± 7.[illegible]	36.7 ± 8.3	38.0 ± 4.8	38.[illegible] ± [illegible].6
Hb_a	13.31 ± 0.81	13.43 ± 1.04	13.2[illegible] ± 0.79	13.42 ± 0.87
$(a-dv)\ O_2$	93.2 ± 1[illegible].8	113.1 ± 18.8	110.9 ± 13.0	100.3 ± 17.4
$Lactate_{dv-a}$	0.06 ± 0.13	0.15 ± 0.34	0.06 ± 0.22	0.07 ± 0.22
Lactate	0.62 ± 0.20	0.64 ± 0.20	0.6[illegible] ± 0.20	0.66 ± 0.19
F	59 ± 17	209 ± 39	212 ± 49	21[illegible] ± 49
Q_O	3.7 ± 1.1	21.6 ± 4.1	20.8 ± 4.5	20.9 ± 4.8
$Q_{la\ rate}$	6.0 ± 3.9	38 ± 84	23 ± [illegible]8	26 ± [illegible]1
T		22.7 ± 7.9	22.3 ± 7.[illegible]	21.7 ± 7.7
T_s		2[illegible].1 ± 4.2	24.1 ± 2.7	26.7 ± [illegible]2
A		10.3 ± 0.9	9.7 ± 1.[illegible]	9.6 ± 1.0
Heart rate	[illegible]6.3 ± 8.2	[illegible]9.8 ± 9.8	[illegible]9.3 ± 7.8	[illegible]0.7 ± 6.7

each contraction was 10 20 and 30 kp in the three periods the sequence being randomized by cyclic permutation between the different subjects

Measurements during exercise and calculations from the data were carried out as described in Chapter V The time course for contraction and relaxation was registered and in these experiments the time from the start of contraction to peak tension was denoted as T_1 The time from peak tension until return to zero (T_2) was also measured as was the amplitude of the peak tension (A) T_3 was calculated as the sum of T_1 and T_2

Results

Rest (Table 15)

The values obtained in this series for forearm dimensions and blood analyses at rest were not significantly different from those presented in Table 8

Exercise

Deep venous oxygen saturation (Fig 31 32 Table 15) The oxygen saturation in blood samples from deep forearm veins decreased at the transition from rest to exercise ($p < 0 01$) and then remained fairly constant during exercise At none of the work intensities (expressed as peak tension during contraction) were there significant changes in deep venous oxygen saturation during the

intensities (M ± SD for 10 subjects) A indicates the amplitude of the peak tension during

20 kp			30 kp		
4 min	8 min	12 min	4 min	8 min	12 min
33 7 ± 6 5	34 6 ± 6 0	36 1 ± 6 5	35 9 ± 5 6	37 1 ± 5 9	37 7 ± 5 2
14 06 ± 0 93	13 78 ± 0 90	13 84 ± 0 75	13 98 ± 1 03	13 78 ± 1 05	13 56 ± 1 01
113 0 ± 16 4	114 7 ± 15 8	110 5 ± 16 4	110 6 ± 13 2	110 1 ± 13 8	110 1 ± 13 2
0 90 ± 0 85	0 68 ± 0 54	0 49 ± 0 41	1 82 ± 0 58	0 39 ± 0 56	1 05 ± 0 51
0 68 ± 0 18	0 71 ± 0 21	0 66 ± 0 17	0 88 ± 0 3[illegible]	0 91 ± 0 26	0 93 ± 0 35
255 ± 46	274 ± 51	290 ± 48	401 ± 87	424 ± 76	455 ± 81
26 1 ± 4 [illegible]	28 6 ± 4 6	29 7 ± 4 8	42 4 ± 10 4	44 7 ± 9 1	47 6 ± 8 8
214 ± 200	172 ± 146	131 ± 98	669 ± 260	565 ± 268	484 ± 273
25 3 ± 8 1	23 7 ± 6 9	22 0 ± 5 1	23 4 ± 6 4	25 6 ± 6 1	26 3 ± 9 2
26 5 ± 4 0	27 0 ± 4 0	25 1 ± 3 1	28 3 ± 3 2	27 7 ± 2 4	28 9 ± 2 5
20 3 ± 1 9	20 1 ± 1 6	19 5 ± 1 6	29 7 ± 5 5	29 0 ± 5 1	29 6 ± 5 8
62 9 ± 8 6	63 7 ± 8 8	64 8 ± 9 4	73 8 ± 14 6	73 5 ± 16 8	79 7 ± 18 9

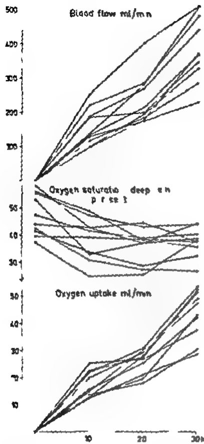


Fig 32 The increase in blood flow and in oxygen uptake from rest to at 12 min exercise and the deep venous oxygen difference as functions of peak tension during isometric exercise

developed (A, kp) during the measurement of blood flow, to the nominal or developed peak tension per unit forearm tissue or to the developed fraction of maximal isometric strength at rest When T values were included with nominal peak tension as independent variables in the regression calculations there was a reduction in the residual standard deviation A significant positive relationship was found between blood flow change and T_1 (regression coefficient b = 6 21***) whereas the regression coefficient for T_2 was not significant For T_4 a negative relationship was found (b = − 4 70**) and for T_s an almost identical but positive relation (b = 4 71**) Similar slightly weaker relationships were found when T values were combined with the other measures of work intensity mentioned above

Oxygen uptake (Fig 31, 32 Table 15 16) The changes in oxygen uptake during exercise approximately followed those of blood flow At exercise with the lowest peak tension no significant increase was found between 4 and 12 min while at 20 and 30 kp exercise this difference was probably significant

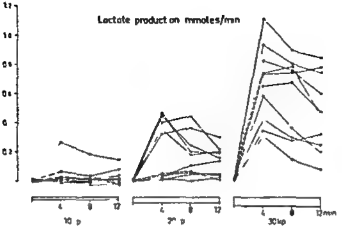


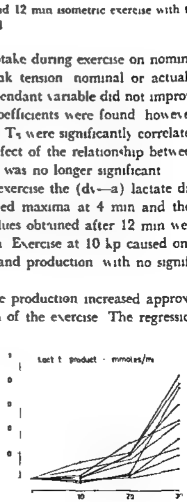
Fig 33 Lactate production at rest and after 4 8 and 12 min isometric exercise with the peak tensions 10 20 and 30 kp Symbols as in Fig 31

The regression of the increase in oxygen uptake during exercise on nominal peak tension was significant The use of peak tension nominal or actually developed per unit forearm tissue as an independant variable did not improve the relationship Slightly higher correlation coefficients were found however when log oxygen uptake was used T_1 T_4 and T_5 were significantly correlated to oxygen uptake After elimination of the effect of the relationship between the T values and blood flow this correlation was no longer significant

Lactate (Fig 33 34 Table 15 16) During exercise the (dv—a) lactate difference and lactate production usually reached maxima at 4 min and then tended to decrease At 30 kp exercise the values obtained after 12 min were highly significantly lower than those at 4 min Exercise at 10 kp caused only very small changes in lactate concentrations and production with no significant differences from the resting values

Both (dv—a) lactate difference and lactate production increased approximately exponentially with rising peak tension of the exercise The regression

Fig 34 The change in lactate production from rest to at 12 min exercise as a function of peak tension during isometric exercise



of log lactate production on nominal peak tension showed a slightly but not significantly closer relationship than that for log lactate concentration difference. Moreover, when the work intensity was expressed as peak tension per 100 ml forearm tissue, a slightly, although not significantly, better correlation was found for log lactate production. Calculations with the peak tension actually developed as an independent variable did not further improve the correlation. T values were not significantly correlated to the (dv—a) lactate difference.

Discussion

The results presented in this chapter were obtained during rhythmic forearm handgrips of certain peak tensions. This has been termed rhythmic isometric exercise although the contraction was maintained for only about half the cycle's duration, whereas the term isometric contraction is usually taken to mean a sustained muscular contraction. Moreover, the term implies constant tension during the activation phase which was not the case in the present experiments since the tension rose rhythmically to a peak value and then declined.

Deep venous oxygen saturation remained approximately constant during rhythmic isometric exercise of different work intensities in agreement with the findings for rhythmic isotonic exercise. The oxygen saturation was found to be slightly higher for isometric than for isotonic exercise. This was probably not caused by an admixture of superficial to deep venous blood. Although not specifically studied for this form of exercise it can be said that if such admixture had occurred it would have resulted in a successive decrease in deep venous oxygen saturation with rising work intensity rather than a constantly elevated level.

Blood flow and oxygen uptake increased in relation to the work intensity in much the same manner as during isotonic exercise. The correlations were slightly closer, however, for log blood flow and log oxygen uptake respectively and work intensity suggesting a tendency towards a curvilinear relation. Both blood flow and oxygen uptake were influenced by the manner in which the exercise was performed. A slow rise in tension (a high T_1) was associated with high blood flow and oxygen uptake and vice versa. The duration of tension release (T_3) had no significant influence on either blood flow or oxygen uptake in contrast to the results for isotonic exercise. It is possible that in rhythmic isometric work of this type blood flow and oxygen uptake are correlated to the work performed in analogy with the relationship between myocardial blood flow and oxygen uptake respectively and tension time index as suggested by Sarnoff *et al.* (1958).

Lactate production was very low at exercise with a peak tension of 10 kp. Although a distinct rise occurred during exercise in some of the experiments the mean for the group did not differ significantly from the mean values at rest. The changes in lactate production during exercise with different peak tensions were similar in type to the findings for isotonic exercise.

The results thus indicate that the pattern of circulatory and metabolic response is similar in rhythmic isometric and in isotonic forearm exercise. The lowest tension developed in the isometric exercise (10 kp) was greater than the tension in the heaviest isotonic exercise (8.3 kp), but the blood flow and oxygen uptake were much higher during the latter. Isotonic exercise at 10 kpm/min (5.6 kp, 30 mm and 60 per min) was approximately equal in intensity to rhythmic isometric exercise at ca. 20 kp peak tension; blood flow was then slightly higher for isometric exercise and the oxygen uptake and lactate production were a little lower. This indicates that the energy requirement for isometric exercise is much smaller than for isotonic exercise at a comparable tension, which is in accordance with the Fenn effect; heat measurements on isolated muscle showed that, compared with isometric contraction, extra energy is liberated when muscles shorten and do work (Fenn 1924). The findings also agree with the observations of Asmussen (1965).

CHAPTER VII

General Discussion

There are various other methods for the quantitative determination of forearm blood flow during exercise apart from the indicator dilution technique used here. The thermodilution method (Fronek and Ganz 1960) was a possibility but at present requires the use of such large catheters that these, when placed in the brachial artery, would probably partially obstruct the blood flow. Venous occlusion plethysmography, which has been used for the determination of calf blood flow during exercise, necessitates manual compression of the supplying artery for a short time during recording (Barcroft and Dornhorst 1949) and would thus invalidate simultaneous metabolic measurements.

The routine procedure adopted for the determination of brachial artery blood flow during exercise appears suitable for the present type of study since it permits repeated quantitative determinations during continuous exercise. The dye solution has to be introduced into the brachial artery through a catheter, but this can be much thinner than in thermodilution experiments and probably does not interfere with the brachial artery blood flow (Pernow and Wahren 1962). The procedure does, however, involve infusion of dye solution at a rate of 34 ml/min. Since this infusion makes up a significant fraction of total flow, especially at low work intensities, it is important to know to what extent it interferes with the initial brachial artery blood flow. From the measurements of venous oxygen saturation and haemoglobin concentration before and during the infusion it is concluded that the infusion simply adds itself to the initial flow within the tested range. The finding of a markedly reduced whole blood viscosity during infusion may provide a clue to how this addition is accomplished. Although the haemodynamic effects of changes in blood viscosity cannot be predicted quantitatively from Poiseuille's law, it is evident that for a given driving pressure a lowered blood haematocrit will tend to increase flow. It is likely that the osmotic balance between blood and tissue was but little disturbed during the infusion, since the osmotic pressure of the dye carrier solution corresponded to that of blood. The infused solution may influence other equilibria and when the technique is used for special purposes the composition of the dye solution may have to be modified to agree, for instance, with the subject's blood glucose or electrolyte concentrations. In the present study the small changes of these concentrations that probably

occurred during infusion were considered to be of little importance The effect on muscle blood flow of changes in potassium ion concentration have been studied by Kjellmer (1965), it is apparent from his results that significant effects on blood flow require considerably larger variations than those which probably occurred in the present study

Blood samples from the deep forearm veins obtained during exercise of a certain minimum intensity contain very small amounts if any of blood from the hand or superficial forearm tissues as is evident from the results presented in Chapter IV This does not mean however that all deep venous blood derives from muscle Approximately 20 per cent of forearm tissue is bone and tendon (Cooper Edholm and Mottram 1955) and although bone tendon connective tissue and fat have a relatively poor vascularity about 10 % of the deep venous blood at rest may come from these tissues (Mottram 1955) During exercise however, this component becomes still smaller and is probably negligible in relation to muscle blood flow

The deep venous oxygen saturation fell at the transition from rest to exercise and then remained approximately constant the values ranging between ca 20 and 40 per cent for different individuals The forearm oxygen consumption during exercise calculated on the basis of blood flow and (a—v) oxygen difference showed that the increase in oxygen uptake was accomplished almost exclusively by a rise in blood flow both at isotonic and at isometric exercise with successively higher but submaximal work intensities Results from animal experiments agree well with the present findings Kramer Obal and Quensel (1939) stimulated the gastrocnemius muscle of the dog via its nerve and measured blood flow and oxygen uptake Both variables increased linearly in relation to the tension developed (isometric contractions) up to 80 per cent of maximal tension after which the blood flow did not increase further The (a—v) oxygen difference was constant up to 80 per cent of maximal tension and then increased maintaining an approximately linear rise in oxygen uptake A similar regulation of oxygen uptake has also been demonstrated for the myocardium (Berglund *et al* 1958 Carlsten *et al* 1961 Messer *et al* 1962) The increased myocardial demand for oxygen during exercise is met by an increased coronary blood flow and a largely unchanged oxygen saturation of coronary sinus blood The mean oxygen saturation given by Messer *et al* (1962) for coronary sinus blood at rest in control subjects (29 per cent) agrees well with the means for forearm deep venous blood during exercise in the present study (Table 10)

The present finding of a constant deep venous oxygen saturation in the forearm at different work intensities seems to be at variance with the finding of a continuous decrease of femoral venous oxygen saturation with increasing

work intensity of bicycle exercise both in healthy subjects at moderate (Carlson and Pernow 1961) and at strenuous exercise (Pernow, Wahren and Zetterquist 1965) and in patients with arteriosclerosis obliterans (Carlson and Pernow 1962) The main explanation of this discrepancy is probably that blood flow in the femoral vein unlike that in the deep forearm veins may contain a varying component of blood from non working tissue The femoral venous oxygen saturation decreases during exercise partly because the component of blood from skin and superficial tissue constitutes a successively smaller portion of total femoral venous flow when muscle blood flow increases at rising work intensity and partly because this component becomes smaller in absolute terms during exercise (Wade and Bishop 1962) Even if the oxygen saturation of muscle vein blood in the leg remains constant during exercise of rising intensity, the femoral venous oxygen saturation will thus decrease successively Moreover, at maximal work intensities on a bicycle ergometer the leg blood flow may be inadequate in relation to the oxygen demand and this may also promote an increased oxygen extraction Accordingly, the femoral venous oxygen saturation was extremely low during exercise at a low work intensity both in patients with reduced cardiac output due to mitral valvular disease (Donald *et al* 1957) and in patients with lowered leg blood flow because of arteriosclerosis obliterans (Carlson and Pernow 1962)

General Summary and Conclusions

The present study is concerned with the quantitative determination of blood flow, oxygen uptake and lactate production in the human forearm during rhythmic exercise in healthy individuals.

1. Forearm blood flow during exercise was determined with an indicator dilution method based on continuous infusion of dye solution (Cardiogreen in 5 per cent dextran solution) into the brachial artery. Satisfactory mixing of dye and blood was achieved within the flow range investigated when the dye was infused at a rate of ca. 3.4 ml/min through a catheter with an internal diameter in the tip of 0.5 mm. The infusion did not alter the blood flow in the brachial artery, the total flow during infusion being equal to the infusion rate plus the blood flow before the infusion. Blood flow determined with this indicator dilution technique agreed well with simultaneous measurements of flow with venous occlusion plethysmography at rest under special circumstances. The coefficient of variation for a single determination of blood flow during exercise was 4.5 per cent.

2. The origin of blood from deep forearm veins was studied during rhythmic exercise. The oxygen saturation in deep venous blood was found to be uninfluenced by changes in superficial venous oxygen saturation induced by body heating. After the injection of contrast medium and dye into the radial artery at the wrist, neither of these indicators was found in significant concentrations in the deep veins when the work intensity was 5 kpm/min or higher. Dye dilution curves were recorded from deep veins after the injection of dye into the brachial artery during exercise. The downslopes of the curves were rectilinear on a semilogarithmic scale. The pO_2 did not differ significantly in blood samples obtained from different levels in the deep veins during exercise. These findings were interpreted to indicate that superficial venous blood from the hand and forearm does not mix to a significant extent with the forearm deep venous blood during rhythmic exercise of a certain minimum intensity. Furthermore, blood flow per unit tissue appears to be the same throughout the region drained by the deep vein.

3. The work intensity of rhythmic isotonic exercise was varied with respect to load (Series 1), frequency (Series 2) and path (Series 3) of contraction. Blood flow and calculated oxygen uptake increased in linear proportion to the

work intensity The rise in oxygen uptake was accomplished by an increase in blood flow, the deep venous oxygen saturation remaining unchanged at different work intensities Both blood flow and oxygen uptake rose to slightly higher values in Series 2 than in Series 3 The results obtained were correlated to the time course of the movement of the hand ergometer handles Both blood flow and oxygen uptake were found to be negatively correlated to the total duration of the contraction and the relaxation as well as to the duration of the relaxation phase alone This finding may imply that a short duration of the relaxation phase was associated with an active engagement of the forearm extensor muscles Lactate deep venous—arterial difference and calculated lactate production both rose approximately exponentially at exercise with successively higher work intensity Lactate deep venous—arterial difference varied inversely with the dimensions of the forearm The mechanical efficiency was calculated, taking into account both the aerobic and the anaerobic lactacid metabolism The values obtained were lower than those for exercise with large muscle groups, for example on a bicycle ergometer

4 During isotonic exercise with the forearm vertically elevated, both blood flow and deep venous oxygen saturation were lower than at exercise with the arm horizontal The oxygen uptake was not significantly different but lactate production was higher during exercise with the forearm vertical

5 During rhythmic isometric exercise with the peak tensions 10 20 and 30 kg of force (kp) the blood flow and oxygen uptake rose in linear proportion to the peak tension, the deep venous oxygen saturation remaining unchanged In contrast to the case at isotonic exercise, long duration of the contraction phase was associated with high blood flow and oxygen uptake Moreover, the duration of the relaxation phase was not correlated to these variables during this form of exercise The pattern of variation for lactate deep venous—arterial difference and lactate production was similar to that for isotonic exercise The results indicate that the energy requirement for rhythmic isometric exercise is smaller than that for isotonic exercise at a comparable tension

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By
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STUDIES ON GLIAL CELLS IN THE HYPOGLOSSAL NUCLEUS OF THE RABBIT DURING NERVE REGENERATION

AND

MORPHOLOGICAL CHANGES IN GLIAL CELLS DURING NERVE REGENERATION

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The present summary is based on the following publications

I Proliferative Changes in Glial Cells during Nerve Regeneration
Sjostrand, J, Z Zellforsch 1965 *68* 481—493

II Morphological Changes in Glial Cells during Nerve Regeneration
Sjostrand, J, Acta physiol scand 1966 *67* Suppl 270 19—43

III Respiratory Enzyme Activities in Neurons and Glial Cells of the Hypoglossal Nucleus during Nerve Regeneration
Hamberger, A and J Sjostrand Acta physiol scand 1966 In press

IV Changes of Nucleoside Phosphatase Activity in the Hypoglossal Nucleus during Nerve Regeneration
Sjostrand, J, Acta physiol scand 1966 In press

These papers will be referred to in the text by their Roman numerals

CONTENTS

INTRODUCTION

In recent years much attention has been given to the metabolic inter relationships between nerve cells and their surrounding glia Biochemical changes in neurons during several different experimental situations have been associated with concomitant changes in the surrounding glial cells (Hamberger 1963 Hyden 1964 Hyden and Lange 1966) During regeneration following crush or section of the hypoglossal nerve extensive biochemical and morphological changes have been described in the hypoglossal neurons (Brattgard *et al* 1957, Takano 1964) In the present investigation interest was focused specifically upon the glial changes which had been observed in the hypoglossal nucleus during this same regenerative period in a preliminary investigation (Sjostrand 1965)

The use of the regenerating hypoglossal nucleus of the rabbit for the study of glial reactions has several advantages The first of these is that the morphology of the hypoglossal neurons is satisfactorily defined (van Gehuchten 1897) The hypoglossal nucleus is well delimited with respect to the surrounding nuclei (Barnard 1940 Torvik and Brodal 1954) Furthermore chromatolysis of neurons in surrounding nuclei of the medulla oblongata has not been observed after section of the hypoglossal nerve (Barnard 1940) Nor has any evidence for axonal connections between the hypoglossal nuclei been found (Green and Negishi 1963) and axon collaterals may be absent (Porter 1965) It is an additional advantage that nerve cell death is not recorded after crush of the rabbit hypoglossal nerve where it traverses the digastric muscle (I) because this would seem to eliminate glial proliferation in scavenger function Finally the hypoglossal nuclei are suitably located for isotope studies employing intracisternal administration (Sjostrand 1965)

Four main problems concerning the effect of nerve crush upon the glial cells in the hypoglossal nucleus were studied

1 The extent of glial cell proliferation by the different types of glial cells and the mode of cell division (mitosis versus amitosis) in the regenerating hypoglossal nucleus have been investigated by radioautography using H^3 thymidine (I)

2 The morphological changes in astrocytes oligodendrocytes and micro glial cells during the glial reaction have been described with the aid of metallic impregnation methods (II)

3 The cellular changes have been characterized with respect to succinoxidase activity Isolated single nerve cells and glial cell samples have been analyzed with Zeuthen s (1953) microdiver technique (III)

4 The different glial cells have been characterized histochemically with respect to certain respiratory enzymes (III) and hydrolytic enzymes (IV)

RESULTS AND DISCUSSION

Cell proliferation in the hypoglossal nucleus after nerve crush

Earlier work has shown that DNA synthesis occurs in neuroglial cells in normal adult brain but only very few mitoses or none at all could be found outside the subependymal layer (Smart and Leblond 1961, Altman 1963, Noetzel and Rox 1964) This discrepancy between the number of glial cells synthesizing DNA and the number of mitotic figures in the normal central nervous system has been discussed in the literature and different interpretations have been given, such as the presence of amitosis (Smart and Leblond 1961, Noetzel and Rox 1964) and DNA synthesis without subsequent cell division (Pele 1963)

Radioautographic technique employing H^3 thymidine (I) and different staining methods for glial cells were used (I, II) to determine the extent of mitotic proliferation in the different glial cell populations during the retrograde reaction after nerve crush

It became evident that glial cells in the adolescent rabbit brain can react by proliferation also during conditions in which direct traumatic injury is absent (I) During the second to fourth day after nerve crush the number of H^3 thymidine labelled glial cells increased tenfold as compared to the unoperated side concomitantly with the appearence of mitotic figures These mitosis had the same scattered distribution as the labelled glial cells and the changes in mitotic index were generally in proportion to the changes in glial labelling index (I Fig 1)

In actively proliferating mammalian tissues outside the central nervous system the labelling index divided by the mitotic index is around 10 for a time interval between injection and sacrifice comparable to that used in this study (Schultze and Oehlert 1960 Messier and Leblond 1960) The same value is 7 4 for glial cells (I) indicating that approximately the same time relation exists between synthesis and mitosis as in other actively proliferating systems No evidence for amitosis or DNA synthesis in glial cells without subsequent mitosis could therefore be observed in

the hypoglossal nucleus following nerve crush Although the occurrence of polyploidy or polyteny has not been excluded it is probable that mitotic activity accounts for all or most cells labelled in this radioautographic study These results indicate that the mitotic figures belonged for the most part to glial cells and that the glial labelling with H^3-thymidine was an expression of mitotic proliferation in the glial cell population

The second problem concerned the type of cells proliferating during nerve regeneration Nuclear characteristics (Penfield 1932) of the labelled cells almost all of which were in a premitotic stage (I) revealed that labelled nuclei outside the vessels were predominantly of microglial type with a tendency for pericapillary localization (I) In addition increased numbers of microglial cells were observed during nerve regeneration in sections impregnated with Del Rio-Hortega s silver carbonate method for microglia (II) and in sections incubated for nucleoside phosphatases (IV) An increase in the number of microglial cells during the retrograde reaction after nerve injury has also been reported by Rapoš and Bakos (1959) and Cammermeyer (1965 a)

During the period with increased labelling of glial cells labelled nuclei and mitotic figures were seen in endothelial cells and pericytes of the vessels in the regenerating hypoglossal nucleus On the second day the microglial proliferation was already marked while the proliferation in the vascular walls was just beginning (I Table 5) On the fourth or fifth day efter nerve crush the proliferative activity of the vascular walls increased (I) at the same time that dark staining nuclei resembling histiocytic nuclei (Kershman 1939) were occasionally observed in juxtavascular positions (II) During this later period of proliferation it therefore seems likely that some proliferating cells seen in the intervening tissue between neurons and capillaries may have been derived from histiocytes of the vascular walls No evidence was found in the hypoglossal nucleus that all or the majority of extravascular mitotic cells observed during nerve regeneration are of histiocytic origin as has been suggested recently by Cammermeyer (1965 a) on the basis of their juxtaposition to vessels

The proliferative activity of astrocytes and oligodendrocytes was lower than for microglial cells during the whole proliferative period as judged from the percentage of labelled cells which show nuclear characteristics for these cells (I)

In conclusion evidence has been presented that glial cells react with mitotic proliferation during the retrograde reaction in the hypoglossal nucleus after nerve crush and that this proliferation concerns mainly the microglial cell population

Morphological and histochemical changes of microglial cells after nerve crush

During the regenerative period conspicuous morphological changes occurred in the microglial cells (II) These changes in microglial morphology were similar to those described as characteristic for the early stages of the microglial cell reaction in various pathological conditions (Del Rio Hortega 1930 1932) The reactive microglial cells had enlarged cell bodies and typically thickened processes The significant microglial increase in projection area, i e the number of hits on microglial cells in per cent of the total number of points counted on the operated compared to the unoperated side in sections (II) indicated an increase in volume of the microglial cell population on the regenerating side During the height of the microglial reaction, i e from the second to the fourth week after nerve crush, a conspicuous increase of microglial cell processes in contact with or in close proximity to the neurons and the capillaries was observed (II, IV)

A prominent increase in the nucleoside phosphatase activity was found in the reactive microglial cells in the histochemical study (IV) after fixation over night in cold formalin It was also observed that the only glial cell type containing nucleoside di- and triphosphatases under normal conditions as well as during the glial reaction was the microglial cell This localization of enzymes hydrolyzing nucleoside di and triphosphates to the microglial cell population was based on the morphology of cell processes, perikarya and nuclei of the enzymatically active glial cells (IV) All active glial cells showed the morphological characteristics of micro glial cells formulated by Del Rio Hortega (1932) Since no evidence of enzymatic activity in astrocytes or oligodendrocytes was found in the medulla oblongata by light microscopy, it was concluded that only the microglial cells contain nucleoside phosphatases after fixation in cold formalin A similar localization of nucleoside phosphatase enzyme activity to microglial cells has been observed in the cerebral cortex and the spinal cord of the rabbit (Sjostrand unpublished observations)

These results do not agree with the conclusions of previous studies which have suggested nucleoside phosphatase activity in astrocytes or oligodendrocytes Barron and Tuncbay (1964) demonstrated nucleoside phosphatases in glial cells of the feline spinal cord which on the basis of vascular attachments were identified as astrocytes Torack (1965) observed nucleoside phosphatases under the light microscope mainly in cells in rat cerebrum which were identified as oligodendrocytes because of a more dense cytoplasm and fewer dendritic processes in electron micrographs

(Torack and Barrnett 1963) These discrepanies may be due to different interpretations of the cell type involved or may be due to species differences also In addition nucleoside phosphatases were found by electron microscopy in parts of astrocytic membranes (Torack 1965) which would be difficult to resolve with the light microscope

Little is known about the function of microglial cells in the central nervous system under normal conditions or about the role of microglial cells in reactive states of the central nervous system which are associated with small or negligible degenerative processes After nerve crush microglial contacts with the neurons and other cell structures are conspicuously increased by hypertrophy of the microglial cell processes and by increase in cell number (II) in a phase during which biochemical studies indicate an increased metabolism of the regenerating neurons (Brattgard *et al* 1957) Because of the absence of significant degenerative processes in the hypoglossal nucleus (I) phagocytosis of degenerated material seems unlikely as a major microglial function in this case Watson (1965) has shown that the proliferative changes in the glial cells are without relation to the extent of nerve cell death during nerve regeneration

There is some experimental evidence for activities other than phagocytosis in reactive microglial cells The demonstration by Klatzo *et al* (1962) of microglial uptake of fluorescein labelled proteins in brain edema indicates that microglial cells may have the capacity to ingest material by a process of pinocytosis Pinocytotic vesicles have been observed in microglial cells both in normal (Andres 1964 Sulzmann 1963) and pathological conditions (Blinzinger and Hager 1962) Since nucleoside phosphatase activity demonstrable after fixation with cold formalin has been associated with active membrane transport both in the central nervous system (Torack and Barrnet 1963) and other organs (Novikoff *et al* 1962) it is suggested that microglial cells also participate to an increased extent in uptake and transport perhaps involving pinocytosis during the regenerative period Additional support for the presence of an increased pinocytotic activity during nerve regeneration is the demonstration of a marked increase in the number of small vesicles in satellite glial cells surrounding *regenerating spinal ganglion cells (Cervos Navarro 1962)*

In summarizing it is found that the volume occupied by microglial cells is increased on the operated compared to the unoperated side and increased numbers of microglial cell processes can be demonstrated in close proximity to neurons and capillaries Increased nucleoside phosphatase activity is observed in the microglial cells and it is inferred that microglia participate to an increased extent in uptake and transport during the regenerative period

Changes of astrocytes after nerve crush

Astrocytic changes previously reported to occur during nerve regeneration (Cammermeyer 1955, Rapoš and Bakoš 1959, Sjostrand 1965) were investigated with respect to proliferation (I), morphology, staining (II) and histochemical characteristics (III IV)

Results showed astrocytic hypertrophy and an increase in volume of the astrocytic cell population in the hypoglossal nucleus on the operated compared to the unoperated side Electron microscopical observations demonstrating astrocytic membranes enveloping the capillaries and in intimate structural contact with the neurons have suggested that astrocytes and their membranes have an important role in transport processes of the mammalian central nervous system (De Robertis and Gerschenfeld 1961, Wolff 1963 Mugniani and Walberg 1964) If this suggestion is valid the increased contact areas of the hypertrophic astrocytes with other cell structures, as judged under the light microscope, support the possibility of increased transport to and from the astrocytes during nerve regeneration In agreement with such an interpretation electron micrographs have demonstrated accentuated astrocytic connections with cytoplasmic vesicles of regenerating hypoglossal neurons intruding into adjoining astrocytic processes (Takano 1964) Electron microscopy has also shown an increased surface area of reactive astrocytes in gliosis (Hager and Blinzinger 1965)

The markedly increased stainability of hypertrophic astrocytes after prolonged formalin ammoniumbromide fixation during the second and third week after nerve crush (II) indicates that a cytochemical change has occurred in the astrocytes Takano (1964) has reported an increase in granular endoplasmic reticulum and fibrils of astrocytes in the hypoglossal nucleus of mice after nerve section The complete reversibility of the astrocytic reaction after nerve crush demonstrated that this glial reaction was not concerned with the formation of permanent scar tissue and this fact point to other roles for the reactive astrocytes (Rubinstein *et al* 1962 Friede 1962)

Incorporation of H^3 thymidine in cells with astrocytic nuclear morphology indicated proliferative changes in the astrocytic cell population The low percentage of labelled astrocytes demonstrated that the number of proliferating astrocytes was low in comparison to the number of proliferating microglial cells (I)

The conspicuous astrocytic hypertrophy observed in this study of regeneration (II) was not associated with any observable increase in NADH$_2$-reductase activity of the astrocytes (III) In contrast to this a striking increase of NADH$_2$-reductase activity has been demonstrated in hyper

trophic astrocytes in white matter after different types of damage to the brain (Friede 1962 Rubinstein *et al* 1962) There may be fundamental differences between astrocytes in gray and white matter or hypertrophic astrocytes may react differently to different experimental conditions

In conclusion the present study has demonstrated that the astrocytes actively participate in the retrograde cell reaction after nerve crush and a prominent astrocytic hypertrophy was observed It is suggested that the hypertrophy of the astrocytes increases the possibilities of interchange and transport to and from the astrocytes during nerve regeneration

Changes in the vascular system of the hypoglossal nucleus after nerve crush

The first observable sign of a reaction in the vascular system of the hypoglossal nucleus after nerve crush was a mitotic proliferation of endothelial cells and pericytes on the second to fifth day (I) During the second to third week an increase in capillarization was observed (IV) In *contrast to the mitotic activity observed in the capillaries of rabbits* (I Watson 1965 Cammermeyer 1965 b) no signs of an increased mitotic activity of the capillaries have been found during regeneration in mice and rats (Watson 1965, Cammermeyer 1965 a)

Concomitant with the increased capillarization of the regenerating hypoglossal nucleus increased activity of nucleoside di and triphosphatases was demonstrated in the capillary walls (IV) Nucleoside di and tri phosphatases have been localized to the basement membrane or to pinocytotic vesicles of endothelial cells in the mammalian central nervous system by electron microscopy (Torack 1965) and the increase in capillary enzyme activity observed in this study may be due to changes in these sites In accordance with this interpretation electron microscopical studies have shown increased numbers of vesicles and invaginations of the endothelial cells and pericytes of the hypoglossal nucleus during nerve regeneration in mice (Takano 1964)

It is known that newly formed blood vessels are more permeable than their mature counterparts (Schoefl 1963) The blood brain permeability for albumin labelled with a fluorescent marker was investigated during the two first weeks after crush of the hypoglossal nerve when new capillaries were being formed No evidence of a break down of the blood brain barrier system for labelled albumin was found (II) Tsang (1940) has reported an increased vascular permeability during retrograde degeneration of thalamic nuclei after experimental lesions of cerebral cortex but in this retrograde reaction degenerative changes were marked

In conclusion, it has been demonstrated that new capillaries are formed in the regenerating hypoglossal nucleus during the first week after nerve crush and that the capillaries after the first week show increased activity of nucleoside di- and triphosphatases

Changes of succinoxidase activity in the hypoglossal nucleus after nerve crush

The respiratory enzyme activity in nerve cell bodies after axonal injury has previously been studied with histochemical methods and with bio chemical methods on homogenates Some authors have claimed an increase (Kreutzberg 1963 Harkonen 1964) while others have reported a decrease in activity of neuronal respiratory enzymes (Howe and Flexner 1947, Friede 1959) during nerve regeneration Howe and coworkers (1945 1947) measured succinic dehydrogenase and cytochrome oxidase activity on homogenates of the anterior grey columns from the operated and un operated side using the Warburg technique They found a decrease in activity of these enzymes during the retrograde reaction An important consideration in biochemical studies of nervous tissue however, is that in a given tissue sample containing a neuronal group the largest volume is generally occupied by glial cells The earlier studies by Howe and coworkers (1945, 1947) have considered glial cells as constituting only a kind of supporting tissue with negligible reactive changes during nerve regeneration

The object in the present study was to reinvestigate respiratory enzyme activity during nerve regeneration and to analyze neurons and glial cells separately since the reaction of the neurons and the glial cells may be entirely different Measurements were made with Zeuthen s (1953) micro diver technique on isolated nerve cells and on glial samples containing surrounding glial cells corresponding in volume to about two hypoglossal neurons (III)

The succinoxidase activity of the glial samples was increased on th operated side of the hypoglossal nucleus from the sixth to the 48th day with a peak of activity on the sixth day (III Fig 3) During the same period the increase in succinoxidase activity per regenerating nerve cell was marked and much higher than in the glial cells (III Fig 1) The peak of glial succinoxidase activity in the regenerating hypoglossal nucleus on the sixth day is in accordance with the morphological (II) and histochemical (IV) observations which indicate that the glial changes are most rapid at this time Since succinoxidase is known to be located in mitochondria it is interesting to note that during the initial period of

regeneration an increase in mitochondrial number and size was revealed by electron micrographs of satellite cells surrounding regenerating spinal ganglion cells (Cervos Navarro 1962) Andres (1961) demonstrated similar findings and the frequent occurrence of cytoplasmic indentations 0 2—0 5 μ long from the satellite cell into the nerve cell during regeneration

The extent of contribution from the different glial cell populations to the increase in glial succinoxidase activity is difficult to determine but it seems probable that all the different types of glial cells contribute to the increase in succinoxidase activity of the glial samples Even though no observable morphological changes have been demonstrated in the oligo dendrocytes in the present investigation (II), it seems probable that the oligodendrocytes are activated since electron microscopy has revealed increases of cytoplasmic organelles and extended cell processes of the oligodendrocytes during nerve regeneration in mice (Takano 1964)

The *un*operated side of the hypoglossal nucleus also demonstrated increase over controls in succinoxidase activity of neurons and glial cells (III Fig 1, 3) In this regard Brattgard and Daneholt (unpublished observations) have recently demonstrated changes in RNA base ratio of the neurons on the unoperated side after crush of the hypoglossal nerve The changes on the unoperated side occur without any visible morphological changes in the neurons (van Gehuchten 1897, Takano 1964) or in the glial cells (II IV) and the nature of this reaction on the unoperated side is unknown

In summarizing the present investigation has demonstrated increase in succinoxidase activity of neurons and glial cells both on the operated and unoperated side of the hypoglossal nucleus The glial increase in succin oxidase activity was most marked on the sixth day in the regenerating nucleus These results provide experimental support for the presence of biochemical changes in the glial cells as well as the neurons during the regenerative period

COMMENTS ON THE GLIAL CELL REACTION AFTER NERVE CRUSH

It is not within the scope of the present paper to give a complete description of the glial role during the retrograde nerve cell reaction However the results in the present investigation give some information about the glial changes and may invite some speculations as to the inter relation between the nerve cell and the surrounding glial cells during nerve regeneration

Regeneration periods The discussion of changes in the nerve and glial cells is facilitated if the regeneration period is divided into latent, out growth and maturation periods (Brattgard *et al* 1957)

1 *The latent period* During the first two days after nerve crush the volume and the RNA content per nerve cell remain largely constant (Brattgard *et al* 1957) At the end of the latent period the first signs of chromatolysis can be seen (van Gehuchten 1897 Brattgard *et al* 1957) concomitantly with the beginning of an increased glial cell proliferation (I, Watson 1965)

2 *The outgrowth period* During this period, from 2 or 3 days to about two weeks after nerve crush, thin new axons grow out and reach the motor end plates The neurons show extensive chromatolytic changes in addition to an increase in cell volume (van Gehuchten 1897, Brattgard *et al* 1957) and in nucleolar volume (Andres 1961, Cervos-Navarro 1962 Watson 1965) The nuclear membrane displays folding (Pannese 1963, Takano 1964) and increased numbers of neurofilaments (Takano 1964) mitochondria and dense bodies (Hudson *et al* 1961, Takano 1964) have been reported Increased incorporation of labelled amino acids has been found in regenerating neurons (For review Rhodes *et al* 1964) and increasing amounts of proteins and RNA have been observed in re generating hypoglossal neurons (Brattgard *et al* 1957) All these findings indicate that an increased synthesis of proteins is occurring in the nerve cell bodies during this period

At the same time mitotic proliferation of glial cells is marked, especially during the first days of the outgrowth period, and increased numbers of perineuronal glial cells are formed (I II Watson 1965) The astrocytes show hypertrophic changes with increased connections to the neurons and capillaries and conspicuous reactive changes in morphology and an in crease in nucleoside phosphatase activity are found in the microglial cells (II IV) Electron microscopy has revealed increased numbers of organelles in perineuronal glial cells (Cervós-Navarro 1962, Takano 1964) and a more intimate structural contact between nerve and glial cells during this period (Andres 1961 Takano 1964)

The capillaries show proliferative changes during the first part of the outgrowth period and after the first week increased capillarization and an increase of capillary nucleoside phosphatase activity can be observed (I, IV) Electron microscopy has demonstrated signs of intensified pin ocytotic activity at the same time (Takano 1964)

3 *Maturation period* During this period from 12 days to up to one year after nerve crush the diameter of the newly established axons increases

and the diameter before nerve crush is restored within one year (Gutmann and Sanders 1943) The full number of terminal branches in the motor end plates is attained about 90 days after nerve crush (Gutmann *et al* 1942)

After the second week the restoration of the Nissl substance begins in the perinuclear area (van Gehuchten 1897 Brattgard *et al* 1957) and the neuronal changes return towards normal during the following months (Takano 1964 Watson 1965 Brattgard *et al* 1957) Concomitantly with the recovery of the neurons the perineuronal glial cells return to the normal glial cell pattern (II IV) Watson (1965) has shown that the number of perineuronal glial cells reached normal values after the first month

Adaptive glia reaction It has been estimated that to regenerate a new axon requires an amount of material equal to fifty times that contained in the cell body (Brattgard *et al* 1957) At the same time that neuronal changes indicate a marked increase in protein synthesis of the regenerating neurons that is during the outgrowth and the first part of the maturation period the glial cell changes are also most pronounced This similarity in time courses of the neuronal and glial reactions strongly suggest that the nerve and glial cell changes are interrelated and that the glial cells take an active part in the adaptive reaction after axonal injury The changes of the glial cells observed in the present investigation have been interpreted as morphological histochemical and biochemical expressions of an intensified transport function

Friede and van Houten (1962) have found that the number of peri neuronal glial cells is related to the axonal length and they assumed that nerve cell bodies with longer axons had a larger need of auxiliary metabolic units Similarly the number of perineuronal glial cells during nerve regeneration may be proportional to the synthetic efforts of the regenerating nerve cells The increased number of cytoplasmic organelles in the glial cells (Cervos Navarro 1962 Takano 1964) points to an aug mented metabolic activity and is compatible with an intensified active transport function

Final conclusion The observations presented here make it clear that the retrograde cell reaction after nerve crush involves in addition to changes in the nerve cell a marked response of glia surrounding the nerve cell body far removed from the site of injury This glial reaction is interpreted as an important adaptive reaction to axonal injury The simultaneous time courses of the neuronal and perineuronal glial reactions strongly suggest a close interaction between nerve cell and glial cells The perineuronal glial cells activated by the axonal injury probably play an important role in transport between the vascular system and the perikaryon of the regener-ating nerve cell

SUMMARY

The effect of nerve crush upon the glial cells in the hypoglossal nucleus of the rabbit was studied with respect to proliferative and morphological changes Attempts were made to characterize some biochemical and histochemical aspects of these changes

The following observations were made

1 A tenfold increase in the number of glial cells incorporating H^3-thymidine occurred during the 2nd to 4th day after nerve crush

2 H^3-thymidine labelled glial nuclei were predominately of the microglial type Low proliferative activity was seen in cells with oligodendrocytic and astrocytic nuclear morphology

3 Mitotic cell proliferation accounted for all or most of the glial cell labelling

4 Microglial cells showed typical morphological changes with thickened cell processes and enlarged cell bodies

5 Activity of nucleoside di- and triphosphatases after fixation in cold formalin was observed among glial cells only in the microglial cells During the retrograde cell reaction after nerve crush an increased nucleoside phosphatase activity was observed in the microglial cells

6 Conspicious hypertrophic changes were observed in the astrocytes The astrocytic reaction was completely reversible

7 Mitotic cell proliferation was observed in the capillaries during the first week After the first week an increased capillarization and an increased capillary activity of nucleoside phosphatases were demonstrated

8 Increases in succinoxidase activity of neurons and glial cells were observed both on the operated and unoperated side of the hypoglossal nucleus A peak of glial activity in the regenerating hypoglossal nucleus was found on the sixth day

9 The glial changes are discussed and the glial reaction is interpreted as an important adaptive reaction to axonal injury The simultaneous time courses of the neuronal and glial reaction strongly suggest a close interaction between nerve cell and perineuronal glial cells The glial cells activated by the distant axonal injury probably play an important role in transport between the vascular system and the perikaryon of the regenerating nerve cell

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MORPHOLOGICAL CHANGES IN GLIAL CELLS DURING NERVE REGENERATION

By

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INTRODUCTION

Glial changes have been described as a characteristic morphological feature of different types of injury to the central nervous system (Linell 1929, Penfield 1932, Lewis and Swank 1953, Klatzo *et al.* 1958). Since brain tissue has been damaged in these cases the glial reaction has often been considered to be associated with the formation of protective scar tissue in the injured brain region. Recent studies, however, point to other roles for the reactive glia (Rubinstein *et al.* 1962, Friede 1962) although the exact nature of the reaction is unknown.

Extensive proliferative changes in the glia surrounding the regenerating motor neurons have been described to occur during nerve regeneration in radioautographical studies (Sjostrand 1965 a, 1965 b; Watson 1965). Morphological changes of the astrocytes and the microglial cells have also been reported during nerve regeneration (Rapoš and Bakoš 1959, Sjostrand 1965 a). These data indicate that the glial cells participate in the retrograde reaction.

The aim of the present study is to make, with the aid of metallic impregnation methods, an extended study of the glial changes previously reported (Sjostrand 1965 a and b). The reacting glial cell populations will be identified and described at various times after nerve crush in a system, the hypoglossal nucleus, in which no appreciable retrograde degeneration seems to occur (Sjostrand 1965 b) and in which cytochemical studies have shown marked changes of the reacting neurons (Brattgard *et al.* 1957, Hamberger and Sjostrand 1966).

MATERIAL AND METHODS

Material

A total of 140 albino rabbits were used for the experiments. Under pentobarbital anesthesia the right hypoglossal nerve was crushed twice with a pair of forceps cooled to —70°C at a point where it traverses the digastric muscle. The animals were killed at different times from 1 to 180 days after nerve crush.

Fixation by immersion

The animals were made unconscious by an air embolus injected into the ear vein and the carotid vessels were cut in order to ensure minimum venous congestion of the brain. Slices of the part of the medulla to be studied were fixed immediately by immersion in formalin-ammonium-bromide at room temperature for 1 to 2 days before staining for astrocytes, formalin-ammoniumbromide for 3 days for microglial cells and in 10 per cent neutral formalin for 2 to 3 days for oligodendrocytes.

Fixation by perfusion

The animals were anesthetized with pentobarbital and heparin was injected intravenously. After thoracotomy the vessels were perfused through a cannula inserted in the ascending aorta. A two-step perfusion procedure (Koenig *et al.* 1945, Cammermeyer 1963) was used with a perfusion pressure of 120 cm water and with formalin ammoniumbromide in the second perfusate in the astrocytic and microglial series and with 10 per cent neutral formalin in the oligodendrocytic series. The dissection was carried out immediately after the perfusion and tissue slices were postfixed in formalin-ammoniumbromide for 13 days before staining for astrocytes, in formalin-ammoniumbromide for 2 to 3 days for microglial cells and in 10 per cent neutral formalin for 2 to 3 days for oligodendrocytes.

Sectioning technique

After fixation the material was frozen with dry ice and cut in a cryostat (Dittes Duspiva) at —20°C with a nominal section thickness of 28 μ.

Metallic impregnation methods for glial cells

Astrocytes. The astrocytes were stained according to the gold chloride sublimate method of Cajal (Romeis 1948 § 1849). The material included two series with different fixation schedules.

Cajal series 1: Fixation by immersion in formalin ammoniumbromide for 1 to 2 days.

Cajal series 2: Fixation by perfusion of formalin ammoniumbromide and postfixation by immersion for 13 days before staining.

The staining was carried out at 26°C except for some sections in the two series which were stained at a higher temperature i.e. 35°C and 40°C.

Oligodendrocytes. The material fixed by perfusion or immersion in 10 per cent neutral formalin was stained for oligodendrocytes according to Tsujiyama's method for oligodendrocytes (Tsujiyama 1963).

Microglial cells The material fixed by perfusion or immersion in formalin ammoniumbromide were stained for microglial cells according to Del Rio Hortega s silver carbonate method for microglia (Romeis 1948 § 1864)

Other staining procedures

Animals were perfused with 10 per cent neutral formalin and the brain stem segments containing the hypoglossal nuclei were postfixed in formalin for more than 2 days and thereafter dehydrated and embedded in paraffin Sections, 5—10 μ thick were stained with toluidine blue cresylviolet, P A S McManus Feulgen (Romeis 1948 Pearse 1960) or luxol fast blue cresyl violet (Kluver and Barrera 1953) for myelin As a control for the metallic impregnation methods used above some material was stained with Del Rio Hortega s silver carbonate method for astrocytes and oligodendro cytes (Romeis 1948 § 1859 and § 1861)

Definition of glial cells

The different types of glial cells were defined according to the morpho logical criteria of normal and reactive glial cells given for astrocytes by Ramon y Cajal (1914) for microglial cells by Del Rio Hortega (1930 1932) and for oligodendrocytes by Penfield (1932)

Semi quantitative analysis of the glial changes

Changes in astrocytes and microglial cells on the operated and unoperated sides were estimated by a point counting method (Hennig 1958) using a Zeiss integration ocular I containing 25 random points Contact of the image of each point with a clearly observed image of a glial cell or its processes at any focal level in the section was called a hit The number of hits on astrocytes or microglial cells as a per cent of the total number of points counted was called the projection area for that type of cell Because of the small diameters of the glial processes no volumetric analysis could be carried out (Hennig 1958) Sections stained with Cajal s gold sublimate method for astrocytes were counted with a 40 × objective and sections stained with Del Rio Hortega s silver carbonate method for microglia, with a 100 × immersion objective The number of testing points counted per section on each side of the hypoglossal nucleus was 200 for Cajal series 1 120—200 for Cajal series 2 and 400—500 in the microglial series The sections to be counted were always chosen randomly within a length of the hypoglossal nucleus extending from the rostral opening of the central canal to a level situated one millimeter more caudally

Blood brain barrier study

The blood brain barrier permeability was studied at different times up to the 14th day after nerve crush. According to the technique described by Steinwall and Klatzo (1965), the animals were injected intravenously with a solution containing 1 per cent Evans blue (Merck) bound to 5 per cent bovine albumin. The animals were killed by exsanguination under pentobarbital anesthesia one or 4 hours after beginning of the injection. After fixation in formalin and sectioning in the cryostat, sections of medulla oblongata were mounted in 50 per cent aqueous glycerol and observed by fluorescence microscopy. The tracer dye, Evans blue, fluoresces bright red under the fluorescence microscope.

Statistical analysis

The significance of the differences between the values obtained by the point counting method for the different glial populations on the operated and unoperated side in the same sections was tested by analysis of variance (Bonnier and Tedin 1962).

RESULTS

Evaluation of the staining methods

The results of the staining with Cajal's gold sublimate method varied with fixation time and fixation method as reported by Ramon y Cajal (1916). After fixation by immersion in formalin ammoniumbromide for 24 to 48 hours as in the Cajal series 1, all types of astrocytes were stained. If the immersion was prolonged from two to 13 days a decreased staining of astrocytes of the protoplasmic type was noted as compared to the fibrous or reactive astrocytes which still stained well.

In the Cajal series 2 the fixation procedure including perfusion and immersion fixation for 13 days resulted in a low staining of normal astrocytes, no protoplasmic astrocytes were stained in this series. The reactive astrocytes, however, showed prominent staining in series 2, indicating a changed and increased affinity for gold during the first four postoperative weeks. The reactive astrocytes were maximally stained in series 2 during the second and third postoperative weeks. Staining at 26° C was found to be optimal in series 1 and 2.

The staining results after the silver carbonate methods for oligodendroglia and microglia did not show any definite variation with the different

fixation schedules used The staining results obtained with Del Rio Hortega s silver carbonate method for astrocytes were found to be the same as these using Cajal s gold sublimate method for astrocytes During the second to fifth week the reactive microglial cells showed a decreased stainability with the silver carbonate method Since the hypertrophic astrocytes to some extent were stained also with Del Rio Hortega s silver carbonate method for microglial cells selective and distinct staining results could not be obtained for the microglial cells during this period

Evaluation of the point counting method

The accuracy of the point counting method was tested in series 1 by counting either 100 or 200 points per section in the hypoglossal nucleus Table I shows the values obtained with 100 or 200 testing points per nucleus and section The variation between sections of values obtained by the point counting method for the different glial populations was calculated at various times after nerve crush (Table II and III)

The staining results were found to be sufficiently reproducible from section to section and from animal to animal in the same series to prove suitable for a qualitative and semiquantitative study of the glial reactions The variation in values between sections in the same animal was small enough to allow the conclusion that the number of counted points used in this study was sufficient to give an accurate measurement of the pro jection areas for the glial cell populations on the operated and unoperated side (Hennig 1957)

Table I

The number of hits on astrocytes in Cajal series 1 as per cent of the total number of testing points with 100 or 200 points counted per hypoglossal nucleus in each section Five sections were scanned in each animal Objective 40 × Zeiss integration eyepiece 1 Mean values ± S E M

Days after nerve crush	Number of animals	Total number of testing points per nucleus per section	Number of hits on astrocytes as per cent of the total number of testing points	
			operated side	unoperated side
9	3	100	18 3 ± 1 4	5 3 ± 1 2
9	3	200	16 6 ± 1 0	4 9 ± 0 8

Table II

Difference of variation between counting five or ten sections in one animal at various times after nerve crush in Cajal series 2 The number of hits on astrocytes on the operated side is expressed as per cent of the total number of testing points Cajal s gold sublimate method for astrocytes Objective 40 × Zeiss integration eyepiece 1

Mean values ± S E M

Days after nerve crush	Number of sections counted	Number of testing points per ocular field	Total number of testing points	Number of hits on astrocytes as per cent of the total number of testing points on the operated side
4	5	100	1063	4 0 ± 0 7
	10	100	2195	4 2 ± 0 4
9	5	50	656	12 3 ± 0 4
	10	50	1293	11 2 ± 0 6
28	5	50	630	3 4 ± 0 9
	10	50	1215	3 1 ± 0 5

Table III

Number of hits on astrocytes and microglial cells on operated and unoperated sides expressed as per cent of the total number of testing points per hypoglossal nucleus and section In all animals 5 sections were counted Objective 40 × in Cajal series 1 and 100 in the microglial series Zeiss integration eyepiece 1

Mean values ± S E M

	Number of days after nerve crush	Number of testing points per nucleus per section	Percentage of hits on the glial cells		P value for difference between the two sides
			operated side	unoperated side	
Series I	9	200	14 7 ± 0 7	6 2 ± 1 0	
astrocytes	9	200	18.3 ± 1 5	5 0 ± 0 [illegible]	< 0 001
	9	200	16 7 [illegible]	3 5 ± 0 [illegible]	
Microglial	4	500	3 [illegible]	1 8 ± 0 [illegible]	< 0 001
series	4	4[illegible]	[illegible]	2.5 ± [illegible]	
	48	[illegible]	[illegible]	[illegible]	
	48	[illegible]	[illegible]	[illegible]	

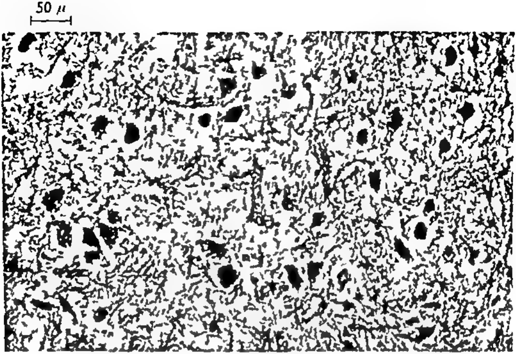
Effect of nerve crush on the astrocytes in the hypoglossal nucleus

Unoperated side On the unoperated side no definite changes could be seen in the astrocytes compared to the astrocytes in the hypoglossal nuclei of control animals The hypoglossal nucleus on the unoperated side and in control animals contained astrocytes of fibrous and protoplasmic type and transitional forms between these types All these types of astrocytes were stained in series 1 In series 2, however, the majority of the astrocytes on the unoperated side had a low stainability

Operated side On the second to third day after nerve crush a slight hypertrophy of the astrocytic cell bodies was observed in series 1 on the operated side of the hypoglossal nucleus This initial astrocytic change was uniformly distributed throughout the regenerating nucleus and affected all parts of the nucleus Between the third and ninth day the astrocytic hypertrophy increased conspicuously and on the ninth day considerable astrocytic hypertrophy could be seen (Figs 1 2 3) Between the ninth and 28th day the astrocytic reaction was on its highest level During this period the reactive astrocytes showed an uniform hypertrophy with thickened processes and footplates which formed an almost complete envelope along all vessels including capillaries (Fig 4) On and after the 28th day the astrocytic reaction diminished and by the 60th day the histological picture was almost normal in this respect with exception of a few slightly hypertrophic astrocytes frequently located in the lateral part of the nucleus

The cytological changes of the astrocytes in series 1 reported above were confirmed by analysis with the point counting method (Fig 5) On the ninth day the projection area occupied by astrocytes in the hypoglossal nucleus on the operated side of the section showed an increase by about 250 per cent as compared to the unoperated side the difference was highly significant ($P < 0.001$) (Table III)

In series 2 primarily astrocytes were stained which had a high affinity to gold Astrocytic hypertrophy and increased stainability could be demonstrated on the fourth day, and from the fourth to the ninth day progressive increases in hypertrophy and stainability were seen The astrocytic changes were prominent between the ninth and 14th day as illustrated in Figure 6 By the 28th day the stainability of the astrocytes had decreased considerably and only a few hypertrophic astrocytes could still be seen Quantitative analysis of the astrocytic changes with the point counting method showed the same general changes as described above in qualitative terms (Fig 7) If one compares the quantitative results



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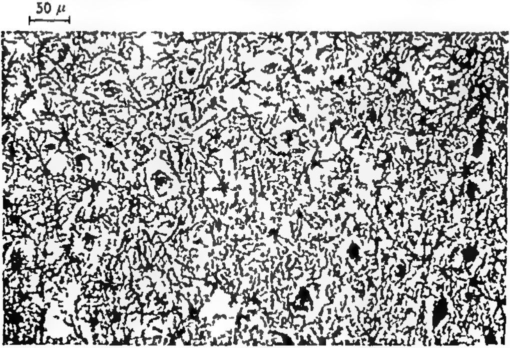
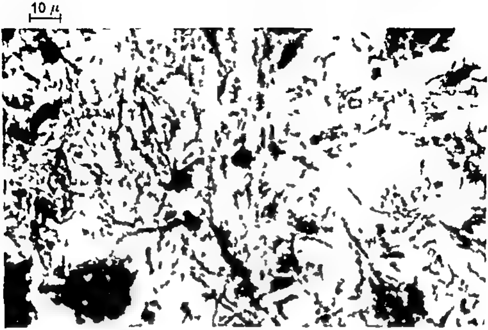
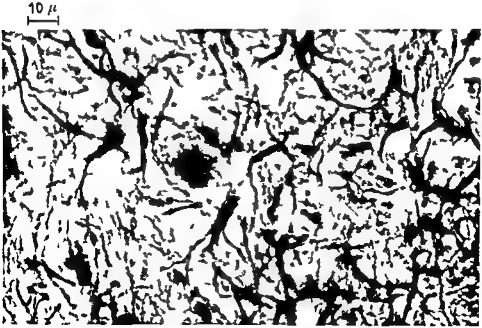


1 b

Fig 1 Hypoglossal nucleus 9 days after nerve crush Cajal's gold sublimate method for astrocytes series 1 fixed by immersion a) unoperated side b) operated side Notice the hypertrophic astrocytes on the operated side

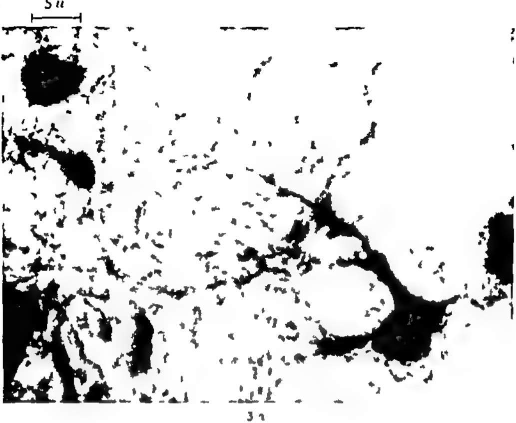


2 a



2 b

Fig 2 Same animal as in fig 1 at a higher magnification a) unoperated side b) operated side



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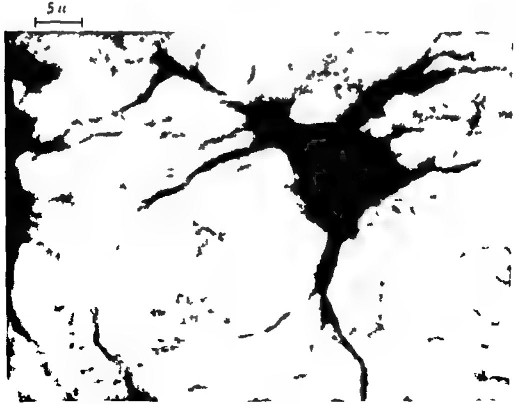


3 b

Fig. 3 Hypoglossal nucleus 9 days after nerve crush Cajal's gold sublimate method

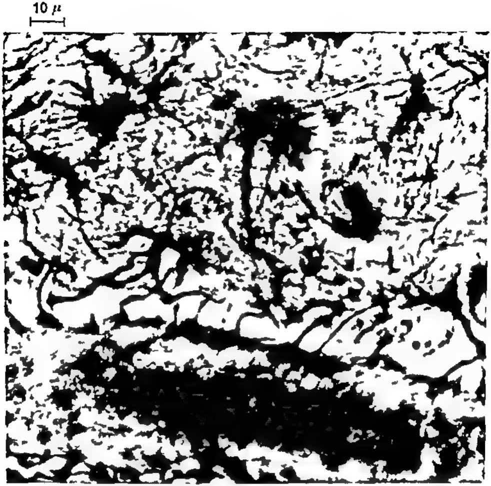


Fig 4 Vessel covered by hypertrophic astrocytic footplates Hypoglossal nucleus 9 days after nerve crush. Cajal's gold sublimate method for astrocytes series 1 operated side

obtained in Cajal series 1 and 2 one can see that the relative changes are parallel in the two series until the ninth day By the 28th day, however a definite difference is seen between the values in series 1 and 2 indicating that the astrocytes were still hypertrophic but that the increased stainability was disappearing with fixation schedules used in series 2

As a control to check that the astrocytic reaction was completely reversible, two animals were studied in series 1 and three animals in series 2 on the 90th day after nerve crush In both series no or negligible changes could be observed and therefore it can be concluded that the astrocytic reaction after nerve crush is completely reversible

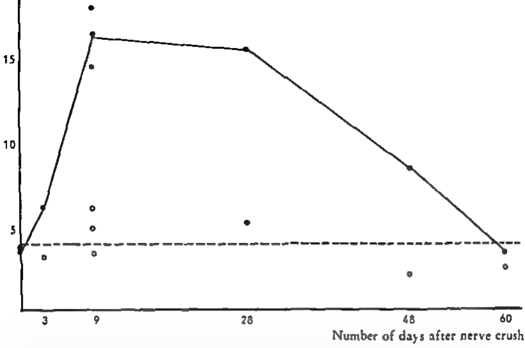
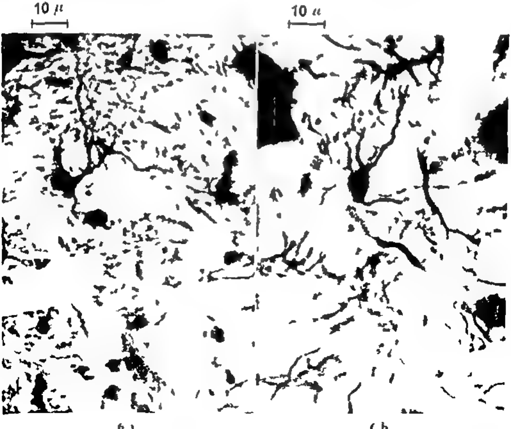


Fig 5 The number of hits on astrocytes as per cent of the total number of testing points on the operated (●) and unoperated (○) side of the hypoglossal nucleus at different times after nerve crush in series 1 200 testing points counted per nucleus and section 5 sections were counted in each animal Cajal s gold sublimate method for astrocytes Solid line connects mean values for the operated sides and interrupted line mean values for the unoperated sides



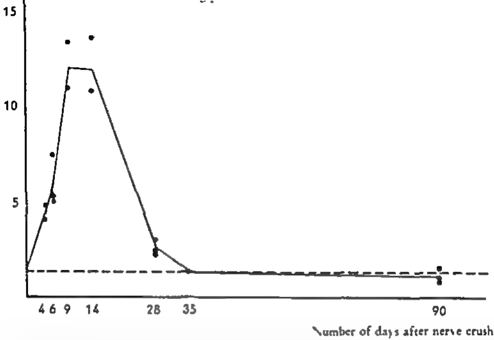
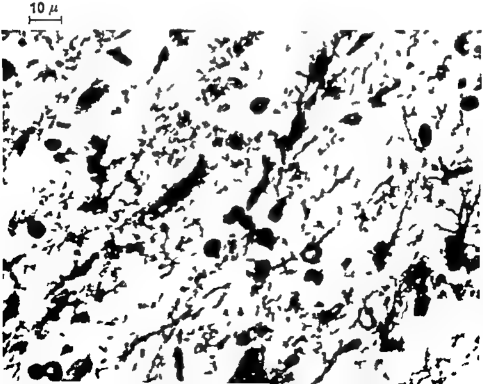


Fig 7 The number of "hits" on astrocytes as per cent of the total number of testing points on the operated side (●) of the hypoglossal nucleus at different times after nerve crush in series 2 Each value represents more than 500 counted testing points Cajal's gold sublimate method for astrocytes after prolonged fixation Solid line connects mean value for the operated side at each postoperative time Interrupted line is an expression of the mean value obtained for the unoperated sides in six randomly chosen animals in this series



Effect of nerve crush on the oligodendrocytes in the hypoglossal nucleus

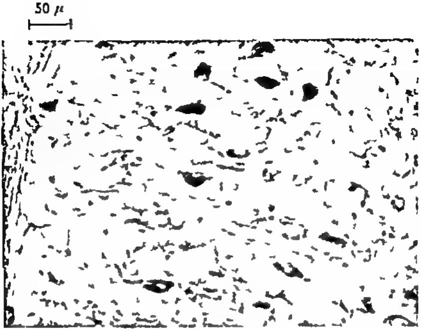
In the normal hypoglossal nucleus, the oligodendrocytes are often arranged in clusters or rows in both perivascular and perineuronal positions During the period of observation from the third to the 36th day no obvious changes could be seen in the oligodendrocytes on either side (Fig 8)

Effect of nerve crush on the microglial cells in the hypoglossal nucleus

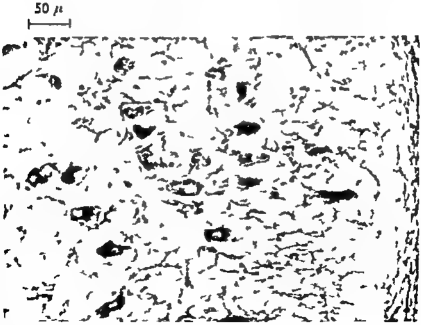
Unoperated side No definite changes of morphology or cell number were observed in the microglial cell population on the unoperated side compared to the hypoglossal nuclei of control animals The unchanged hypoglossal nucleus contained a sparse number of microglial cells scattered throughout the nucleus and microglial cells were seen in close proximity both to capillaries and neurons (Figs 9 a, 10 a)

Operated side After the second or third day reactive changes were seen in the microglia on the operated side (Fig 11) The reactive microglial cells had enlarged cell bodies and thickened processes (Fig 11 b) In contrast to the uniform reaction of the astrocytes the microglial reaction was patchy during the initial period of regeneration (Fig 9 b) The areas containing reactive microglial cells had a tendency to be localized along the larger vessels in the peripheral parts of the nucleus This patchy microglial reaction was especially marked on the third and fourth day in the ventromedial part of the nucleus (Fig 11 c) At the same time as these nests of reactive microglial cells were seen chromatin rich, dark staining nuclei were occasionally observed in the perivascular tissue in sections stained with ordinary histological methods These dark staining nuclei were only seen on the third to fifth day in the regenerating hypoglossal nucleus On the 14th day the patchy reaction had been replaced by a diffuse general microglial reaction with reactive microglial cells throughout the whole nucleus, and this type of reaction still remained on the 28th or 35th (Fig 11 d) day After the first week an increase in number of microglial cells was seen both in silver impregnated sections and in sections stained with nuclear stains By the 48th day the morphology of microglial cells had largely returned to normal During the whole period of the microglial reaction a large number of the reactive microglial cell bodies were found in contact with the vessels with their cell processes touching both capillaries and neurons

The projection area occupied by microglial cells measured with the point-counting method was significantly larger ($P < 0.001$) on the operated side

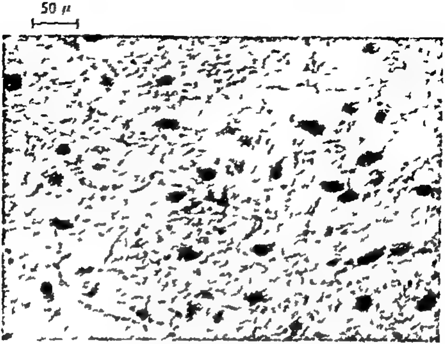


9 a



9 b

Fig 9 Microglial cells in the hypoglossal nucleus 4 days after nerve crush a) unoperated side b) operated side Del Rio Hortega's method for microglia



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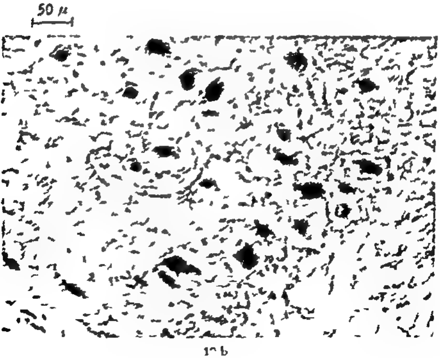
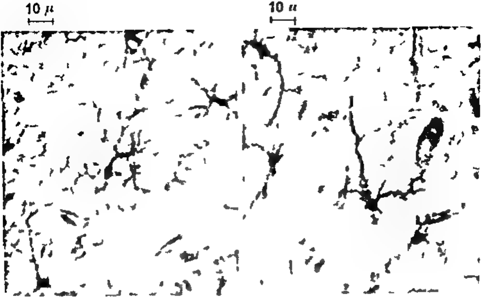
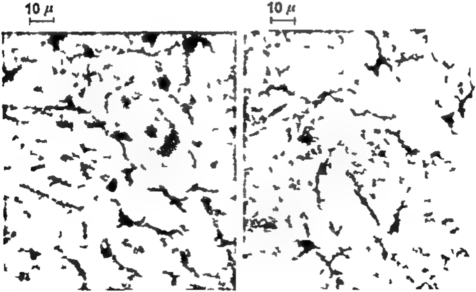


10 b

Fig. 10. Microglial cells in the hypoglossal nucleus 4 days after nerve crush. a) unoperated side b) operated side. Del Rio-Hortega's method for microglia



11 a. 11 b



11 c 11 d

Fig 11 The morphology of the microglial cells in the hypoglossal nucleus after nerve crush Del Rio-Hortega's method for microglia a) Unoperated side 35 days after operation b) Operated side 4 days after operation c) Operated side 4 days after operation. d) Operated side 35 days after operation

as compared to the unoperated side on the fourth and 48th day (Table III) Between the 14th and 28th day no point-counting was carried out because of a decreased selectivity of the staining method for the microglial cells when the astrocytic stainability was at maximum After the 48th day there was a decrease in microglial reaction and after the 90th day only a slight reaction could be seen On the 180th day no differences were observed between operated and unoperated side

Effect of nerve crush on the blood brain barrier in the hypoglossal nucleus

No signs of a break-down of the blood brain barrier for labelled albumin could be found in the hypoglossal nucleus on the operated or unoperated side from the time of operation to the 14th day after nerve crush The *area postrema* could be observed in the same sections and in this region labelled protein was found outside the lumina of the vessels in all animals as described by Klatzo *et al* (1962)

DISCUSSION

The present paper reports a marked hypertrophy of the astrocytes surrounding the regenerating neurons after nerve crush The initial phase of this astrocytic reaction occurring from the third to the 14th day, is similar to the changes of astrocytes observed after injury to the central nervous system (Linell 1929 Penfield 1932, Lewis and Swank 1953, Klatzo *et al* 1958) and during nerve regeneration in mice (Rapoš and Bakoš 1959)

In the case studied here the reactive astrocytes return towards normal cytology after the seventh week and by the 60th day they do not differ from those of controls This is in contrast to the persistent astrocytic reaction after brain injury (Klatzo *et al* 1958) and nerve section (Sjostrand unpublished observations) The complete reversibility of the astrocytic reaction after nerve crush demonstrated that this glial reaction is not concerned with the formation of permanent scar tissue and this fact points to other roles for the reactive astrocytes (Rubinstein *et al* 1962 Friede 1962)

Results showed astrocytic hypertrophy and an increase in volume of the astrocytic cell population in the hypoglossal nucleus on the operated compared to the unoperated side Electron microscopical observations demonstrating astrocytic membranes enveloping the capillaries and in intimate structural contact with the neurons have suggested that astrocytes and their membranes have an important role in transport processes of the

mammalian central nervous system (De Robertis and Gerschenfeld 1961 Wolff 1963 Mugniani and Walberg 1964) If this suggestion is valid the increased contact of hypertrophic astrocytes with other cell structures as judged under the light microscope suggests the possibility of increased transport to and from the astrocytes during nerve regeneration In agreement with such an interpretation electron micrographs have demonstrated accentuated astrocytic connections with cytoplasmic vesicles of regenerating hypoglossal neurons intruding into adjoining astrocytic processes (Takano 1964) Electron microscopy has also shown an increased surface area of reactive astrocytes in gliosis (Hager and Blinzinger 1965)

The markedly increased stainability after prolonged fixation of hypertrophic astrocytes during the second and third week after nerve crush indicates that a cytochemical change has occurred in the astrocytes Takano (1964) has reported an increase in endoplasmic reticulum and fibrils of astrocytes in the hypoglossal nucleus of mice after nerve section

The conspicuous hypertrophy of astrocytic footplates found in the present study and the mitotic proliferation of endothelial cells reported previously (Cammermeyer 1965 Sjostrand 1965 b) indicate that a change takes place in the cells and cell membranes interposed between nerve cells and the blood Electron microscopy has given corresponding results *showing increased numbers of vesicles and invaginations of the plasma* membranes in the endothelial cells of the capillaries during nerve regeneration (Takano 1964) There is no evidence in the present study, however of a break down of the blood brain barrier for labelled albumin during the first two weeks after nerve crush of the hypoglossal nerve Tsang (1940) has reported an increased vascular permeability during the retrograde degeneration of thalamic nuclei after experimental lesions of cerebral cortex but in this retrograde reaction the degenerative processes are marked compared to their negligible presence in the regenerating hypoglossal nucleus

Cammermeyer (1960) suggests that oligodendrocytes are exclusively situated along vessels in most parts of the brain but this does not appear to be the case in the hypoglossal nucleus During the period of extensive changes in the astrocytes and microglial cells no clear changes were observed in the oligodendrocytes In brain injury regressive changes have been described in oligodendrocytes (Penfield and Cone 1926 Cone 1928 Lewis and Swank 1953) In mice changes in oligodendrocytes have been observed in silver-impregnated sections during the retrograde reaction (Rapoš and Bakoš 1959) but in this species a marked nerve cell degeneration has also been found after section of the hypoglossal nerve (Watson

1965) Perhaps injury after nerve crush of the hypoglossal nerve in rabbits is not severe enough to cause changes in oligodendrocytes which would be observable in the light microscope (Sjostrand 1965 b) Electron microscopy has revealed increases of cytoplasmic organelles and extended cell processes of oligodendrocytes during nerve regeneration (Takano 1964)

The microglial hyperplasia observed in the present study is confirmed by the radioautographical observation that the majority of the H^3-thymidine labelled cells found during the second to fifth day were of microglial type (Sjostrand 1965 b) Since most of these labelled cells had typical microglial nuclear morphology (Del Rio-Hortega 1932) and were in a premitotic stage it can be concluded that the main mitotic proliferation occurs in the microglial cell population

Reactive microglial cells appear after the third day in a patchy distribution around the larger blood vessels At the same time chromatin-rich dark staining nuclei are found occasionally in juxtavascular positions This concomitant appearance of dark staining nuclei and nests of reactive microglial cells around the vessels resembles the picture in the prenatal or newborn rabbit, in which microglia invade the brain (Del Rio Hortega 1932, Kershman 1939, Rapős and Bakős 1959) Since the common histogenetic view is that the microglial cells are of mesodermal origin (Del Rio Hortega 1932, Kershman 1939) it is probable that some of the reactive microglial cells are derived from the histiocytes in the adventitia of the vessels The possibility that some microglial cells can even have their origin from circulating blood cells cannot be excluded since Konigsmark and Sidman (1963) have demonstrated that the majority of brain macrophages are derived from blood cells in brain injuries

After the first week the reactive microglial cells are distributed throughout the nucleus and their cell bodies are often seen in the vicinity of capillaries In view of the initial scattered microglial reaction observed by radioautography this later change from a patchy to a more uniform morphological distribution can as well be explained by earlier more extensive morphological changes in microglial cells localized along larger vessels compared to the other microglial cells as by a migration of the reactive microglial cells throughout the hypoglossal nucleus

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SUMMARY

Changes of glial cell morphology were studied with metallic impregnation methods in the hypoglossal nucleus of rabbits after nerve crush. The glial changes consisted of an astrocytic reaction and a microglial cell reaction.

The astrocytic reaction began on the second or third day with slight astrocytic hypertrophy uniformly distributed throughout the hypoglossal nucleus. This hypertrophy increased up to the ninth day at which time the astrocytic reaction was at its maximum. Between the ninth and 28th day the astrocytic hypertrophy was prominent. A marked increase in stainability after prolonged fixation in formalin ammoniumbromide was seen in the reactive astrocytes during the second to third week. An increase in the projection area of the astrocytes on the operated compared to the unoperated side from the fourth to the 28th day was demonstrated by a point counting method. During the same period the capillaries traversing the regenerating hypoglossal nucleus were covered by enlarged astrocytic processes and footplates. After the 28th day the astrocytic reaction diminished and on the 90th day the astrocytes had reassumed their original morphology.

The microglial reaction started on the second to fourth day with reactive microglial cells localized around the larger vessels. On and after the 14th day the microglial reaction was generalized with reactive microglial cells with decreased stainability throughout the nucleus. During the height of the reaction microglial hyperplasia was seen. After the 48th day the microglial reaction decreased and on the 180th day the microglial cells had returned to normal appearance.

The blood brain barrier system showed no alterations in permeability for labelled albumin during the regenerative period.

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II RNA changes in rabbit cerebellar Purkinje cells after caloric stimulation and vestibular neurotomy

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From the Department of Neurobiology University of Göteborg Sweden

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CHAPTER I

RNA changes in rabbit cerebellar Purkinje cells and in cells from the lateral vestibular nucleus after unilateral cold and warm water irrigation

INTRODUCTION

The role of the cerebellum as part of the neurovestibular unit and of the vestibulo-ocular reflex pathway has been the subject of numerous investigations The cerebellum influences ocular movements both directly and indirectly through the vestibular nuclei and thus modifies the ocular vestibular reflexes which have been studied by Chambers and Sprague (1955) Fernandez and Schmidt (1962) and others (for further references see Dow and Manni 1964) This relationship of the cerebellum to vestibular structures is also supported by numerous neuroanatomical investigations showing connections between the cerebellum and the vestibular nuclei (Dow 1936 Larsell 1937 Brodal and Torvik 1957 for review see Brodal Pompeiano and Walberg 1962)

During nervous activity in the cerebellum the RNA content in Purkinje cells shows changes parallelling the functional localization (Jarlstedt 1966 a) Thus the RNA content in Purkinje cells from the vermian part of the lobulus centralis the paraflocculus and the pyramis together with the copula pyramidis significantly increased during a combined extero- and proprioceptive stimulation while the RNA content in the Purkinje cells from the nodulus and the lateral parts of the culmen the simplex and crus I showed no change after this type of stimulation The same areas were also studied after rotatory stimulation and it was found that Purkinje cells from the lobulus centralis and the nodulus but not cells from the pyramis the copula pyramidis and the paraflocculus reacted by increasing their RNA content There were changes not only in total RNA but also in base composition in cells from the vermis of the lobulus centralis and from the nodulus supporting the conclusion that these cells had been influenced by the stimulation

The present study an extension of the vestibular stimulation experiments deals with the effects of cold and warm water caloric stimulation upon Purkinje cell RNA and with the question of whether these effects are also limited to restricted areas of the cerebellar cortex Caloric irrigation affects the impulse activity of the vestibular nerve (Gernandt 1949) and this kind of vestibular stimulation has been used to study vestibular reflexes the cerebellum and the reticular formation being regarded as regu

lators of the vestibulo-ocular reflex arc (Dunlap 1925, Hood and Pfalz 1954, Henriksson Kohut and Fernandez 1961, Fluur and Mendel 1962 a, b) Caloric stimulation has the advantage over rotatory stimulation that tactile and proprioceptive impulses, such as may reach the cerebellum during rotatory experiments, are minimized. It therefore provides a more clear-cut vestibular influence and in addition the effect of the irrigation can be judged from the type of nystagmus produced

The effect of the caloric stimulation upon the RNA content of the small and medium-sized cells from the ventral and dorsal part of the lateral vestibular nucleus was studied also, since primary vestibular fibers reach these cells only in the ventral part of this nucleus (Walberg, Bowsher and Brodal 1958)

MATERIAL AND METHODS

Albino rabbits weighing between 1400—1700 grams were used

Stimulation procedure

Two groups of rabbits were subjected to repeated irrigation in the left outer ear for 30 min daily for seven days One group was irrigated with water at 20°, and the other was irrigated with water at 48° The animals were sacrificed about 24 hours after the last irrigation Two additional groups underwent a single warm or cold water caloric stimulation in the left outer ear for 30 min These latter animals were killed 1 hour after the stimulation period. All animals were anesthetized intravenously by nembutal (0,4 ml/kg body weight = 24 mg/kg body weight) and fastened onto a table. The head was bent backwards in such a position that the lateral semicircular canals were vertically orientated In this position warm water calorization increases the impulse activity in the ipsilateral vestibular nerve whereas cold water irrigation decreases or inhibits the ipsilateral activity (Gernandt 1949) The ears were pulled upwards and fixed to a bar A fine rubber tube (about one mm diameter) was introduced into the outer auditory canal with its tip close to the drum membrane The position of the tip was checked under an operation microscope The water was taken from temperature-regulated containers placed about one meter above the level of the animal The temperature of the water immediately before its entrance into the auditory canal was measured as well as that flowing out of the ear

In some animals a spontaneous nystagmus was evoked by the anesthesia but about 20 to 30 seconds after the beginning of the irrigation a nystagmus resulting from the stimulation was achieved, i e nystagmus to the contralateral side using cold water and to the ipsilateral side using warm water

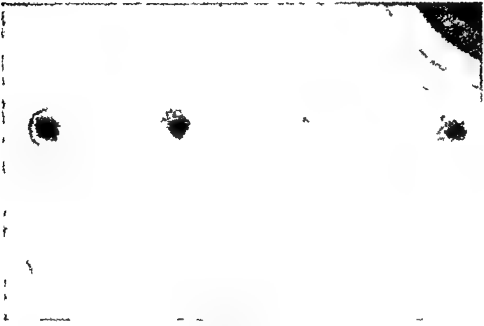

Fig 1 RNA extracts from Purkinje cells photographed at 257 mμ The upper absorption spots contain more RNA than the lower (From the left and right lateral parts of the lobulus centralis one week after vestibular neurotomy on the right side)

RNA determinations

Pieces of the cerebellum and the medulla oblongata were removed immediately after the death of the animal and fixed for 90 min in Carnoy's solution (i e absolute ethanol chloroform concentrated acetic acid 6/3/1 by volume) The pieces of tissue were embedded in paraffin and cut at 50 μ RNA was determined in samples of small — to medium sized cells from the lateral vestibular nucleus and in Purkinje cells isolated by micromanipulation and extracted with ribonuclease in an oil chamber (Edström 1958) The size of the cells from the corresponding areas of the left and right lateral vestibular nucleus was measured to ensure that comparisons were made between cells of the same size The collected extracts were evaporated to dryness on a quartz glass and redissolved in a glycerol-containing

buffer The drops were then photographed together with a reference system in ultraviolet light at a wave length of 257 $m\mu$ (Fig 1) The amounts of RNA in the ultraviolet absorbing spots were determined by a photometric procedure (Edstrom 1964) Other samples of Purkinje cells were extracted in the same way and then the evaporated extracts were redissolved in 4-N hydrochloric acid for hydrolysis and subsequent determination of base composition (Edstrom 1964)

Statistical analyses

The significance of the difference between the left and right sides of the various areas was tested by variance analyses on the homogenity of data within hierarchical subdivisions (Bonnier and Tedin 1940) Significant p values were obtained when the variance between the left and right side was significantly greater than the variances within a side The side differences in individual animals were also subjected to t tests

The results of RNA determinations and base compositions are given in Tables I—VI The mean, standard error of the mean (S E M), number of animals and number of samples are given in the tables In order to make the results more readable the values presented in the tables are the averages of the mean values from the individual rabbits

RESULTS

Lateral vestibular nucleus

The small and medium-sized cells from the ventral part of the ipsilateral lateral vestibular nucleus showed 46 % higher RNA content after a single warm water caloric stimulation compared to those of the contralateral side The cells from the dorsal part of this nucleus in contrast, had the same values bilaterally (Table I)

Purkinje cells

In view of the results from the previous study (Jarlstedt 1966 a), Purkinje cells from the following lobules were analyzed for their RNA content the lobulus centralis the lateral parts of the paramedian lobule, the paraflocculus and the nodulus

Preliminary tests in 5 animals ascertained that no difference in Purkinje cell RNA content existed between the left and right sides of the various lobules in unstimulated rabbits Nor could any difference in cellular RNA content be found between the ventral and dorsal paraflocculus in un

TABLE 1

RNA content in small to medium sized cells from the lateral vestibular nucleus The rabbits were irrigated with warm water in the left outer ear for 30 min Determinations carried out on samples of two cells each Mean values in $\mu\mu g \pm$ S E M

Area	Left side	N*)	n**)	Right side	N	n	Per cent higher on the left side	P value
Dorsal part	231 ± 19	4	27	242 ± 16	4	26	—	Not significant
Ventral part	30[illegible] ± 21	4	30	207 ± 14	4	28	46	$P < 0.001$

*) N represents the number of animals

**) n represents the total number of samples

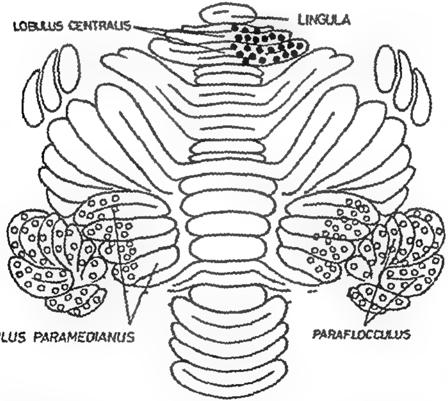

Fig 2. Rabbit cerebellum unfolded in one plane Surface view (Redrawn after Brodal, 1940)

● ● Areas with unilaterally higher Purkinje cell RNA content after cold water irrigation in the left outer ear

○ ▫ Areas with bilaterally similar Purkinje cell RNA content after cold water irrigation in the left outer ear

buffer The drops were then photographed together with a reference system in ultraviolet light at a wave length of 257 mμ (Fig 1) The amounts of RNA in the ultraviolet absorbing spots were determined by a photometric procedure (Edstrom 1964) Other samples of Purkinje cells were extracted in the same way and then the evaporated extracts were redissolved in 4 N hydrochloric acid for hydrolysis and subsequent determination of base composition (Edstrom 1964)

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TABLE III

RNA content in Purkinje cells from rabbits irrigated with cold water in the left outer ear for 30 min. Determinations carried out on samples of two cells each. Mean values in $\mu\mu g \pm$ S.E.M.

Area	Left side	N*)	n**)	Right side	N	n	Per cent higher on the right side	P value
Lobulus centralis vermian part	216 ± [illegible]	2	12	294 ± 12	2	12	23	P < 0.01
Lobulus centralis lateral part	164 ± 16	2	11	225 ± 13	2	12	37	P < 0.01
Paraflocculus	197 ± 11	2	12	220 ± 11	2	12	12	Not significant

*) N represents the number of animals

**) n represents the total number of samples

TABLE IV

RNA content in Purkinje cells from rabbits irrigated with warm water in the left outer ear 30 min daily for seven days. Determinations carried out on samples of two cells each. Mean values in $\mu\mu g \pm$ S.E.M.

Area	Left side	N*)	n**)	Right side	N	n	Per cent higher on the left side	P value
Lobulus centralis vermian part	257 ± 9	5	30	198 ± 11	5	30	31	P < 0.001
Lobulus centralis lateral part	260 ± 13	5	30	221 ± 10	5	30	19	P < 0.001
Paraflocculus	234 ± 14	5	30	248 ± 11	5	30	–	Not significant

*) N represents the number of animals

**) n represents the total number of samples

TABLE V

RNA content in Purkinje cells from rabbits irrigated with warm water in the left outer ear for 30 min. Determinations carried out on samples of two cells each. Mean values in $\mu\mu g \pm$ S.E.M.

Area	Left side	N*)	n**)	Right side	N	n	Per cent higher on the left side	P value
Lobulus centralis vermian part	263 ± 11	2	12	212 ± 9	2	12	23	P < 0.001
Lobulus centralis lateral part	232 ± 16	2	12	156 ± 11	2	[illegible]	48	P < 0.001
Nodulus	228 ± 9	2	12	141 ± 9	2	12	64	P < 0.001
Paraflocculus	233 ± 9	2	23	228 ± 15	2	23	2	Not significant

*) N represents the number of animals

**) n represents the total number of samples

TABLE VI

Composition of Purkinje cell RNA from the left and right sides of the vermian parts of the lobulus centralis and the nodulus The rabbits were irrigated with warm water in the left outer ear for 30 minutes Base ratios expressed as molar proportions in per cent of the sum

Vermis lobulus centralis						
Rabbit	Adenine	Guanine	Cytosine	Uracil	$\frac{\text{Adenine + Guanine}}{\text{Cytosine + Uracil}}$ ± S E M	n*)
1 Left	21 13	32 14	25 96	20 76	1 15 ± 0 05	6
Right	19 64	29 69	28 85	21 83	0 98 ± 0 04	6
2 Left	20 48	32 50	26 02	21 01	1 14 ± 0 07	6
Right	19 86	31,28	26 47	22 41	1 05 ± 0 04	6

P value for the significance of the difference in A+G/C+U quotients between total left and right values P < 0 02

Nodulus						
Rabbit	Adenine	Guanine	Cytosine	Uracil	$\frac{\text{Adenine + Guanine}}{\text{Cytosine + Uracil}}$ ± S E M	n*)
1 Left	20 42	32 58	26 79	20,20	1 13 ± 0 02	3
Right	21 03	30,54	25 64	22 79	1 06 ± 0 02	3
2 Left	21,55	32,15	25 69	20 61	1 16 ± 0 02	3
Right	20 76	30 60	28 83	19 81	1 06 ± 0 05	3

P value for the significance of the difference in A+G/C+U quotients between total left and right values P < 0 01

Control values from the lobulus centralis of three unstimulated rabbits **)					
Adenine	Guanine	Cytosine	Uracil	$\frac{\text{Adenine + Guanine}}{\text{Cytosine + Uracil}}$ ± S E M	n*)
20 65	29 64	27 80	21 92	1 02 ± 0 06	3

*) n represents the number of hydrolyses and each hydrolysis value is the mean of two to five microelectrophoretic separations

**) No difference in base composition exists between the left and right side of control animals

Warm water irrigation

The RNA changes after warm water calorization were the opposite to those observed after cold water irrigation In the lobulus centralis (vermis and lateral part) there was a significantly higher RNA content in Purkinje

cells taken from the ipsilateral side The difference between sides after caloric stimulation once daily for seven days was about 25 % (Table IV and Fig 3) As with cold water irrigation the RNA content in the para flocculur Purkinje cells was the same on both sides (Table IV)

After a single warm water irrigation the RNA content in Purkinje cells from the ipsilateral side of the vermis and hemispheral part of the lobulus centralis was about 35 % higher than the contralateral side (Table V) As can be seen from this table no significant difference with regard to RNA content in Purkinje cells could be detected between the two paraflocculur lobuli The nodulus was also analyzed for Purkinje cell RNA content in this group of animals and the cells from the ipsilateral side had a total

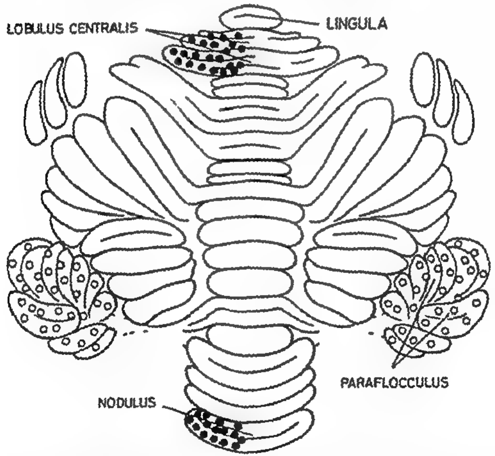

Fig 3 Rabbit cerebellum unfolded in one plane Surface view (Redrawn after Brodal 1940)
● ● Areas with unilaterally higher Purkinje cell RNA content after warm water irrigation in the left outer ear or after right sided vestibular neurotomy
○ ○ Areas with bilaterally similar Purkinje cell RNA content after warm water irrigation in the left outer ear or after right-sided vestibular neurotomy

RNA value 64 % higher than the contralateral side (Table V and Fig 3)

Microelectrophoresis of Purkinje cell RNA from the vermian part of the lobulus centralis taken from rabbits calorized once with warm water showed a significantly higher purine/pyrimidine quotient on the ipsilateral side and the contralateral side had a quotient similar to controls (Table VI) From Table VI it can be seen that there was also the same differences in base ratios between the two sides of the nodulus

DISCUSSION

The localization of the RNA changes after vestibular stimulation of rabbits reported here is in complete agreement with the earlier studies performed on rats (Jarlstedt 1966 a) The results of the RNA determinations in the different areas will be discussed separately

The lateral vestibular nucleus

The marked difference in RNA content between the ventral parts of the lateral vestibular nuclei on each side and the lack of any difference between the dorsal parts are very interesting in the light of the report by Walberg Bowsher and Brodal (1958) that primary vestibular fibers end only in the ventral part of the lateral vestibular nucleus, there making synaptic contacts with the small and medium-sized cells, whereas the giant cells are devoid of primary vestibular nerve endings The findings in the present investigation prove that the small and medium sized neurons react with increased RNA content upon caloric stimulation and that this reaction is localized to the ventral part of the nucleus

The lobulus centralis

From the present cytochemical observations a vestibular influence on the lobulus centralis seems likely These findings are consistent with those of the previous study (Jarlstedt 1966 a) in which Purkinje cell RNA increased in the lobulus centralis after rotatory stimulation The difference in base ratios of Purkinje cell RNA from the left and right sides of the vermian part of the lobulus centralis is a further indication of a definite influence upon this area during vestibular stimulation These results are interesting because no primary or secondary vestibular fibers have been traced to this area It should be emphasized that the present study measured total cell response to stimulation in intact animals The stimulus which gave a cytochemical result may have been relayed via several interconnections which integrated and modulated the stimulus

After stimulation of peripheral branches of the vestibular nerve, Andersson and Gernandt (1954) found a localized response in the caudal portion of the anterior lobe of the cerebellum These responses had a slightly longer duration than those obtained by Dow (1939) from the nodulus which belongs to the so called vestibular part of the cerebellum This indicates a vestibular influence upon the anterior lobe not necessarily mediated by primary or secondary vestibular fibers The question then arises as to which pathways these impulses may follow

Destruction of the vestibular nuclei gives terminal degeneration in the paramedian reticular nucleus (Brodal and Gogstad 1957) This nucleus sends fibers to the anterior lobe and these connections are chiefly homolateral (Brodal and Torvik 1954) It is thus possible that these connections contribute to the cytochemical findings in the lobulus centralis

It cannot be excluded that during the experiment the cerebellum was influenced by other than purely vestibular impulses The caloric irrigation may have stimulated vestibulo-spinal reflexes which in turn increased the cerebellar input of spinal impulses However the spinal impulses mediated by spinocerebellar tracts and also relayed via the inferior olive and the lateral reticular nucleus do not terminate in the lateral most part of the lobulus centralis (cf Jarlstedt 1966 a) Spinal afferents to the paramedian reticular nucleus are scarce (Brodal and Gogstad 1957) and it does not seem likely that spinal impulses via this nucleus could be the main reason for the RNA changes The pons also receives spinal afferents to some extent and projects upon the cerebellar hemispheres The present results however show that the lateral parts of the paramedian lobule and the paraflocculus did not develop any differences between the sides

Impulses from the motorcortex may have also increased during the experiment in spite of the fact that little or no muscular activity was observed during the irrigations The motorcortex sends fibers to the inferior olive the paramedian reticular nucleus and the pons among other areas The connections with the inferior olive and the paramedian reticular nucleus are mostly bilateral (Walberg 1956 Brodal and Gogstad 1957) and accordingly impulses from the motorcortex relayed via these stations could not produce differences between the sides so marked as those noted here Connections between the motorcortex and the pons are chiefly homolateral but as mentioned above parts of the cerebellar areas to which the pons projects did not change their Purkinje cell RNA content after this kind of stimulation Thus the exact pathways responsible for mediating the impulses which cause the Purkinje cell RNA changes in the lobulus

centralis after the stimulation used in these experiments cannot be determined as yet with certainty

During cold water caloric stimulation the efferent activity from the ipsilateral labyrinth is decreased and the contralateral activity increased, with warm water irrigation the ipsilateral activity is increased and the contralateral activity decreased (Gernandt 1949, Fluur and Mendel 1962 a, b) This corresponds well to the findings that after cold water irrigation the RNA content in the Purkinje cells from the ipsilateral lobulus centralis is significantly lower than in Purkinje cells from the contralateral side The results are the same regardless of the number of caloric stimulations This crossed effect may be mediated by connections between the vestibular nuclei on each side It is known that secondary commissural fibers pass between the left and right vestibular nuclei (Rasmussen 1932, Lorente de Nó 1933, Ferraro, Pacella and Barrera 1940) Electrophysiological investigations proved the existence of interconnections between the vestibular nuclei of the two sides (DeVito Brusa and Arduini 1956) Moruzzi and Pompeiano (1957) postulated that the efferent activity from the vestibular nuclei on the side ipsilateral to that subjected to vestibular neurotomy *was further suppressed by inhibitory influences coming from intervestibular neurons of the contralateral nuclei* This so-called crossed labyrinth inhibition since it affects the cerebellar inflow of impulses from *the vestibular nuclei, fits well with the cytochemical findings of a lowered* amount of RNA in Purkinje cells in the lobulus centralis ipsilateral to cold water irrigation

The ipsilaterally higher RNA values in lobulus centralis Purkinje cells after warm water irrigation also agree with the above-mentioned findings of possible ipsilateral impulse pathways and the 'crossed labyrinth inhibition phenomena of Moruzzi and Pompeiano (1957) (see also Fluur and Mendel 1962 a b)

As can be seen in the tables the differences between the two sides is generally greater after a single caloric stimulation than after seven daily irrigations This may be the effect of an habituation of the vestibular nuclei a phenomena well known from studies on habituation of vestibular reflexes (Henriksson Kohut and Fernández 1961, Fluur and Mendel 1962 a, b) An habituation does not normally occur during anesthesia, but the animals used in this study were presumably anesthetized so lightly that a certain amount of habituation did occur The nystagmus frequency and duration of after nystagmus decreased after repeated irrigations occurences which are regarded as signs of habituation These findings are in agreement with the assumption that the cerebellum is a part of the vestibulo-ocular reflex arc

The change of base composition in Purkinje cell RNA from lobulus centralis in rabbits calorized once with warm water shows that the left side with higher RNA contents increased the purine/pyrimidine quotient compared to the contralateral side which had a base composition similar to controls The functional significance of such a base change is still unknown (cf Jarlstedt 1966 a) but can be regarded as additional evidence of an effect of the stimulation upon the Purkinje cells of the lobulus centralis

The paramedian lobule

After seven irrigations with cold water there were no signs of changed RNA content in the Purkinje cells from this area This lobule receives fibers mainly from the pons the externate cuneate nucleus, the olive and the lateral reticular nucleus (for references see Jansen and Brodal 1954, 1958 Grant 1962) The paramedian lobule is considered to receive sensory impulses from the entire body via these relay stations This lobule is also connected with the oculomotor nuclei (Manni Azzena and Dow 1965) and it possibly may have been affected during the experiments However, no asymmetry with regard to RNA content was observed, in agreement with the general opinion that this part of the cerebellum does not have intimate connections with the vestibular apparatus

The paraflocculus

Dow (1936 1939) was unable to find vestibular connections with the paraflocculus The recent observations by Brodal and Drablos (1963) and Brodal and Høivik (1964) give evidence that this area does receive primary vestibular fibers to a small extent Chambers and Sprague (1955) did not find any vestibular dysfunction after ablation of the paraflocculus The finding that the Purkinje cells of the paraflocculus did not react with an RNA increase after vestibular stimulation agrees with the earlier studies performed on rats (Jarlstedt 1966 a)

The nodulus

Of all the areas investigated following calorization the nodulus showed the greatest difference between the left and right sides This could be expected since the nodulus is one of the parts of the cerebellum known to receive almost exclusively vestibular fibers (Brodal Pompeiano and Walberg 1962, Brodal and Høivik 1964) The base composition was also significantly different between the two sides of the nodulus These changes in base quotients are identical to those of the lobulus centralis a further indication of a vestibular influence upon the lobulus centralis

CHAPTER II

RNA changes in rabbit cerebellar Purkinje cells following unilateral vestibular neurotomy

INTRODUCTION

Nystagmus towards the intact side after unilateral vestibular neurotomy subsides after about one month but for several years there is a *preponderance of the nystagmus towards the intact side* (*Aschan, Bergstedt* and Stahle 1956) These clinical observations have been parallelled in animal experiments Hallen and Hamberger (1964) observed that a compensation after unilateral vestibular neurotomy is achieved earlier in rabbits than in human beings Determinations of respiratory enzymes (succinoxidase) confirmed that the vestibular nuclei were affected A significant difference in enzyme activity was noted between the lateral vestibular nuclei two weeks after the operation

The cerebellum plays an important role in the coordination of the activities in the vestibular nuclei (DeVito, Brusa and Arduini 1956, Moruzzi and Pompeiano 1956) After localized injuries to the nodulus a postural nystagmus can be observed which disappears after bilateral labyrinthectomy (Fernández 1960) If the nodulus is destroyed after a bilateral labyrinthectomy there are no changes in the symptoms produced by the previous labyrinthectomy Thus it is concluded that the nodulus acts as an inhibitor of the vestibulo ocular reflex arc An asymmetrical reaction within the cerebellum might therefore be expected after unilateral vestibular neurotomy It was thus of interest to determine by quantitative analyses of Purkinje cell RNA the response of the cerebellum and also the time course of eventual RNA changes in the compensating animal

The purpose of this study was to determine in particular if the lobulus centralis as well as the nodulus, reacted as a part of the vestibulo cerebellar unit during these conditions

MATERIAL AND METHODS

Albino rabbits of both sexes weighing between 1,5—1,8 kg, were used Three groups of animals were investigated at intervals of 1, 2 and 4 weeks after the vestibular neurotomy

Vestibular neurotomy

The operations were carried out in order to give parallel information about the effect of neurotomy upon the cytochemistry of neurons in the lateral vestibular nucleus (Blomstrand *et al.* 1966).

The animals were anesthetized with nembutal (0.4 ml kg/body weight) intravenously. The skull was exposed through a midline incision in the occipital region. The bone was drilled away in a small area and the dura was incised, exposing the medulla beneath the cerebellum. After suction of excess cerebro-spinal fluid the medulla was carefully moved to the left and the nerves in the right internal auditory meatus were exposed. A micro hook was placed around the vestibular nerve which was then divided. A nystagmus of destruction type with the fast phase towards the left ensued immediately after the section. The animals showed other symptoms of disturbed vestibular balance, i.e. torsion of the head to the right and a tendency for rotation towards the right. Within 1 to 3 days the nystagmus and the tendency for rotation subsided but the right torsion of the head persisted. The animals had a good appetite after the operation and were generally found to have increased in weight after the operation.

RNA determinations and *statistical analyses* were performed in the same manner as described in the previous chapter.

RESULTS

The results of the RNA determinations and the base compositions are given in tables VII—X. The mean ± standard error of the mean, number of animals and number of samples are presented in the same way as in chapter I.

Purkinje cells from the lobulus centralis showed a significantly higher RNA content on the contralateral (left) compared to the operated (right) side in both the vermian and hemispherical parts (Table VII and Fig. 3) in rabbits killed one week after the neurotomy. The nodulus also showed a similar difference on the two sides (Table VII). The RNA content in Purkinje cells from the paraflocculus did not differ between one side and the other (Table VII). Table VIII shows the results of microelectrophoretic separations of RNA bases from rabbits killed one week after the operation. A comparison is made between the left and right sides of the lobulus centralis and values from control rabbits are also given. There is a significant difference in the purine/pyrimidine quotient between the left and right sides of operated animals. The values from the right side of the lobulus

TABLE VII

RNA content in Purkinje cells from rabbits killed one week after vestibular neurotomy on the right side Determinations carried out on samples of two cells each Mean values in $\mu\mu g \pm$ S E M

Area	Left side	N*)	n**)	Right side	N	n	Per cent higher on the left side	P value
Lobulus centralis vermian part	258 ± 12	4	21	194 ± 7	4	24	36	P < 0 001
Lobulus centralis lateral part	223 ± 9	4	24	197 ± 10	4	24	15	P < 0 01
Nodulus	214 ± 14	3	18	156 ± 12	3	17	39	P < 0 001
Paraflocculus	191 ± 7	4	24	199 ± 6	4	24	—	Not significant

*) N represents the number of animals

) n represents the total number of samples

TABLE VIII

Composition of Purkinje cell RNA from the left and right sides of the vermian part of the lobulus centralis The rabbits were killed one week after vestibular neurotomy on the right side The values collected from three rabbits

	Adenine	Guanine	Cytosine	Uracil	$\frac{\text{Adenine + Guanine}}{\text{Cytosine + Uracil}}$ ± S-E M	n*)
Left	19 30	27 45	30 77	22 49	0 [illegible] ± 0 04	5
Right	20 90	29 46	28 47	21 17	1 02 ± 0 03	5

P value for the significance of the difference in A+G/C+U quotients between total left and right values P < 0 05

Control values from the lobulus centralis of three unoperated rabbits **)					
Adenine	Guanine	Cytosine	Uracil	$\frac{\text{Adenine + Guanine}}{\text{Cytosine + Uracil}}$ ± S E M	n*)
20 65	29 64	27 80	21 92	1 02 ± 0 06	3

) n represents the number of hydrolyses and each hydrolysis value is the mean of two to five microelectrophoretic separations

*) No difference in base composition exists between the left and right side of control animals

TABLE IX

RNA content in Purkinje cells from rabbits killed two weeks after vestibular neurotomy on the right side Determinations carried out on samples of two cells each Mean values in $\mu\mu g \pm$ S E M

Area	Left side	N*)	n**)	Right side	N	n	Per cent higher on the left side	P value
Lobulus centralis vermian part	243 ± 13	4	24	205 ± 12	4	24	19	P < 0.01
Lobulus centralis lateral part	248 ± 7	3	[illegible]	202 ± 13	3	[illegible]	23	P < 0.001
Paraflocculus	231 ± 13	3	[illegible]	232 ± 12	3	18	—	Not significant

*) N represents the number of animals
**) n represents the total number of samples

TABLE X

RNA content in Purkinje cells from rabbits killed four weeks after vestibular neurotomy on the right side Determinations carried out on samples of two cells each Mean values in $\mu\mu g \pm$ S E M

Area	Left side	N*)	n**)	Right side	N	n	Per cent higher on the left side	P value
Lobulus centralis vermian part	242 ± 10	4	24	198 ± 10	4	24	23	P < 0.001
Lobulus centralis lateral part	245 ± 10	4	24	207 ± 14	4	24	[illegible]	P < 0.01
Paraflocculus	219 ± 11	4	24	224 ± 12	4	24	—	Not significant

*) N represents the number of animals
**) n represents the total number of samples

centralis correspond to control values whereas the purine/pyrimidine ratio [illegible] decreased on the opposite side

In rabbits killed two weeks after the neurotomy a corresponding side difference in the vermian and hemispheral part of lobulus centralis was seen [illegible] higher RNA values on the side contralateral to that of the operation (Table IX) Again in this group no difference was observed between the left and right paraflocculus (Table IX) with respect to RNA content in Purkinje cells As can be seen in Table X the results 4 weeks after the neurotomy were identical with those 1 and 2 weeks after the operation

On no occasion was any difference observed between the ventral and dorsal paraflocculus with regard to RNA content in Purkinje cells No data were obtained from the nodulus in animals killed 2 and 4 weeks after the neurotomy

DISCUSSION

The two earlier studies in this series indicated a vestibular influence on the lobulus centralis as well as on the nodulus (Jarlstedt 1966 a, and Chapter I of the present paper) As judged by RNA determinations the paraflocculus does not react to vestibular stimulation Possible pathways for mediating vestibular impulses to the lobulus centralis have been discussed in Chapter I and by Jarlstedt (1966 a)

A disappearance of spontaneous activity in the ipsilateral vestibular nuclei has been observed clinically in humans who for one reason or another have suffered unilateral labyrinthectomy, while activity in the contralateral nuclei has remained at its normal level (Fluur 1960) Electrophysiological investigations on cats with implanted electrodes (Gernandt and Thulin 1952) showed that after section of the eighth nerve the spontaneous activity in the ipsilateral vestibular nuclei was reduced or totally abolished, whereas the activity in the contralateral nuclei was unchanged This imbalance may be due to the crossed labyrinth inhibition postulated by Moruzzi and Pompeiano (1957) According to this theory the contralateral vestibular nuclei are released after unilateral vestibular neurotomy and suppress the ipsilateral efferent activity via inhibiting intervestibular neurons Thus, impulses to the cerebellum via primary vestibular fibers ipsilateral to the sectioned nerve are totally abolished and ipsilateral impulses via secondary vestibular fibers are also abolished or strongly reduced, which is in good agreement with the findings presented in this work

The RNA content in Purkinje cells from the side of the lobulus centralis and the nodulus contralateral to the vestibular neurotomy was about the same as that found in previously-studied normal material (Jarlstedt 1962) and in control rabbits investigated during the course of this work The values from the operated side were lower than normally found This also compares well with the results obtained in the caloric stimulation studies Cold water irrigation as mentioned in Chapter I abolishes or strongly reduces the efferent activity in the ipsilateral vestibular nerve and in its effect may thus be compared to vestibular neurotomy on the same side

The vestibular neurotomy did not affect the RNA content in para

floccular Purkinje cells in agreement with the earlier findings that neither rotatory nor caloric stimulation changed the Purkinje cell RNA content in this area These findings support the assumption that the paraflocculus is influenced to a relatively small extent by vestibular impulses

Investigation of RNA base composition from Purkinje cells of the lobulus centralis in rabbits killed one week after the neurotomy showed a decrease of the contralateral purine/pyrimidine quotient while the ipsilateral values were the same as in controls Here also this imbalance may be regarded as additional evidence of vestibular action upon the lobulus centralis

DeVito Brusa and Arduini (1956) state that the cerebellum plays an important role in the regulation of the activity in the vestibular nuclei Ito and Yoshida (1964) showed that stimulation of the anterior vermis inhibits the activity in the lateral vestibular nucleus Fernandez Lindsay and Alzate (1959) and Gernandt and Gilman (1959) also consider the cerebellum to be inhibitory on the vestibular nuclei As mentioned above the efferent activity in the vestibular nuclei is diminished ipsilateral to a sectioned vestibular nerve This creates an asymmetry in the cerebellum with a functional overweight for the contralateral side The higher RNA content in the Purkinje cells from the contralateral lobulus centralis and the nodulus may thus also be regarded as due to activity in these cells suppressing the contralateral vestibular nuclei in order to achieve equilibrium between the vestibular nuclei of the both sides

The difference in Purkinje cell RNA content between the operated and intact side persisted unchanged for as long as four weeks after the vestibular neurotomy This suggests that the RNA change represents a long term cerebellar change in the animal compensating after vestibular neurotomy

The following conclusions may be drawn

1 Changes in the activity of the Purkinje cells can be documented by quantitative RNA determinations
2 Stimulation affects the Purkinje cell RNA content in localized areas
3 The Purkinje cell RNA content in the lobulus centralis and the nodulus is influenced by caloric stimulation and vestibular neurotomy in contrast to cells from the paramedian lobule and the paraflocculus

SUMMARY

In Chapter I the effect of unilateral cold or warm water caloric stimulation upon the RNA content in cells from the lateral vestibular nucleus and upon cerebellar Purkinje cells is presented Two groups of rabbits

:dium sized cells from the ventral part of the ipsilateral
nucleus had significantly higher RNA content after a
irrigation compared to the contralateral side The same
the dorsal part of this nucleus had similar values bi-

nt in the lobulus centralis, the paraflocculus, the para-
the nodulus was investigated both ipsilateral and contra-
of the stimulation After warm water caloric stimu
: cells from the ipsilateral lobulus centralis and nodulus
igher RNA content and purine/primidine quotient than
ells from the contralateral side After cold water irri
of the total RNA determinations in Purkinje cells were
ect to the sides affected The paraflocculus had similar
n all cases as did the lobulus paramedianus after seven
rrigation

ie effect of unilateral vestibular neurotomy upon cere
RNA is presented Three groups of rabbits were subjected
ibular neurotomy The animals were killed 1, 2 and 4
eration The RNA content in Purkinje cells from both
is centralis the paraflocculus and the nodulus was in
lobulus centralis and the nodulus the nonoperated side
cantly higher Purkinje cell RNA content compared to
This was found in all three groups The paraflocculus
ies bilaterally in all three groups One week after the
ny the purine/pyrimidine quotient of Purkinje cell RNA
ower on the non-operated side of the lobulus centralis
iperated side The conclusions are drawn that changes
he Purkinje cells can be detected by quantitative RNA
l that Purkinje cells from the lobulus centralis and the
ir RNA content after caloric stimulation and vestibular

ias been supported by grants from the Medical Faculty
eborg

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MEASUREMENTS OF NORMAL AND COLLATERAL CORONARY BLOOD FLOW BY CLOSE ARTERIAL AND INTRAMYOCARDIAL INJECTION OF KRYPTON[85] AND XENON[133]

By

ERLAND LINDER

ABSTRACT

ERLAND LINDER *Measurements of normal and collateral coronary blood flow by close arterial and intramyocardial injection of Krypton[85] and Xenon[133]* Acta physiol scand 1966 69 Suppl 272 5—31

A recent method for measuring collateral blood flow in the myocardium of dogs was based on an analysis of the composite clearance of Krypton[85] administered close arterially (JOHANSSON LINDER and SEEMAN 1964 1965) This method has been critically analysed and various sources of possible errors in the estimations are discussed

Comparisons between paired measurements by this technique and results obtained by recordings of the wash out rate of intramuscularly injected Xenon[133] depots in the well perfused and ischemic myocardial areas respectively strongly suggest that the method is fairly accurate and reliable despite the fact that it has obvious theoretical limitations

The results also reveal that the collateral flow to the ischemic area is not entirely uniform Within a narrow border zone there is a steep reduction of blood flow with a more gradual diminution towards central parts of the ischemic zone This regional heterogeneity in collateral flow could not for several reasons be analysed in exact detail from the composite curve alone but was revealed by recordings of clearance of strictly localized intramuscular tracer injections

Peak flow during reactive hyperemia could also be fairly satisfactorily measured provided certain precautions were taken The results suggest that it is in addition possible to make a fair estimation of the mass of the ischemic tissue from the composite curve

The method may prove to be useful in experimental studies designed to investigate different means for improving collateral blood supply to an ischemic myocardial area

Introduction

In recent papers (JOHANSSON, LINDER and SEEMAN 1964, 1965) a modification of the Krypton[85] clearance method was described which was designed *for quantitative estimation of collateral coronary blood flow (CCBF) to a* myocardial region made acutely ischemic by coronary artery obstruction. A composite clearance curve was obtained during temporary arterial occlusion allowing an estimation both of the collateral capillary blood flow in the ischemic region and of the normal blood supply to surrounding muscle portions. Assuming the proper conditions, the size of the ischemic tissue portion in relation to the well perfused tissue could also be deduced from the clearance curves. Lastly, upon release of the arterial occlusion the ensuing reactive hyperemia produced a rapid wash out of residual isotope activity in the previously ischemic region. It was shown that the peak of this hyperemic flow rate could be determined from the corresponding section of the clearance curve. In general, the respective flow rates could be reproduced with fairly good accuracy when repeated occlusions were performed in these studies, while there was less satisfactory agreement concerning the estimation of the size of the ischemic region contra the well perfused region.

In an analysis involving a graphical solution of a composite clearance curve, where the average flow rates in normally perfused and ischemic muscle sections are represented, a falsely defined slope for the slow component would also lead to an incorrect slope for the fast component. The estimation of the relative size of the ischemic portion versus the normally perfused portion would also be affected. It was therefore considered justified to investigate the validity of the present method in more detail in order to explore its accuracy. The present report deals with a number of these control experiments in which duplicate determinations of the respective flow rates, with evaluations of the size of the ischemic area, have been performed under various conditions where the tracer substance was administered intra-arterially to the region studied. *In addition, the flow values deduced from* such measurements were compared with measurements where the tracer was administered locally by means of small intramuscular injections.

Methods

The data to be reported were collected in experiments on 32 dogs (14 to 52 kg) anesthetized with pentobarbital intravenously. The animals were curarized, intubated and ventilated in the Engstrom respirator with oxygen and nitrous oxide (1:1) without rebreathing. The details of the surgical procedure and the placement of the catheter are fully described in previous

papers (JOHANSSON, LINDER and SEEMAN 1964, 1965). In three experiments, however, the catheter for the isotope injections was placed in a distal position instead with injection of the tracer either before or after the arterial occlusions as described in the first report (1964).

Most animals were heparinized (1.0–2.5 mg/kg) and heparin was always added to the saline solution used to flush the catheters. Repeated measurements of rectal and myocardial temperature were performed during the course of the experiment in most of the animals and the temperature never exceeded the limits 36.5° and 39.5° C. Arterial oxygen saturation, carbon dioxide content and pH were determined at regular intervals in a few animals. Arterial blood was almost fully saturated in all these instances, while CO_2 and pH values indicated a moderate degree of hyperventilation in the animals. Repeated measurements of whole blood viscosity were performed by means of the Brookfield viscometer in several experiments, and showed only small variations. Hematocrit in at least two arterial blood samples was determined with the method of Winthrop in most experiments.

ECG and mean arterial blood pressure were continuously monitored in all the experiments. Surgical A—V block was induced in the great majority of the animals to keep a constant heart rate by artificial stimulation with a Grass stimulator.

The gamma radiation of Kr^{85} or Xe^{133} was monitored by a scintillation detector (2 inch NaI) placed 10 cm inside the opening (5 or 10 cm diam.) of a lead collimator, which was positioned above the anterior wall of the exposed left ventricle. The position of the detector was either kept constant during the entire course of the experiment, or was accurately replaced if temporarily removed for an intramuscular isotope injection.

One or two GEIGER MULLER end window tubes were used (Philips no. 18504) for counting of beta radiation after intraarterial or local administration of Kr^{85}. They were suspended in wide coiled springs barely making contact with the epicardial surface, thus preventing their displacement through heart movements. The detectors' outputs were converted by linear rate meters with time constants of 0.3 to 20 sec. and recorded on potentiometer writers at paper speeds of 2/3 to 2 inch/min. Radioactive tracers were administered either as (1) close arterial injections or (2) topical intramuscular injections.

1) Either Kr^{85} or Xe^{133} (Radiochemical centre, Amersham, England) dissolved in saline, was injected through the coronary catheter in amounts of 0.5 to 1.5 ml to achieve maximal counting rates of gamma activity of 30 000 to 100 000 cpm. When measuring the beta activity of intraarterially administered Kr^{85}, maximal counting rates were kept around 10 000 cpm to

avoid significant coincidence loss in the measured activity — The coronary catheter was always flushed with an equal amount of saline or blood Total injection times ranged from [illegible] to 22 seconds in the majority of experiments

2) Isotope solutions in amounts of 0 02 to 0 [illegible] ml injected directly into the myocardium usually effected maximal counting rates of less than 30 000 cpm for gamma activity and around 10 000 cpm when recording beta radiation The injections were made with a thin needle (0 46 mm O D) inserted into the ventricular wall either perpendicularly to the epicardial surface for 2—4 mm or in an oblique direction for 10—15 mm In the former case a moveable collar on the needle shaft determined the depth of the local injection and hence the position of the tracer depot Thin guide wires and injections of dyed solutions were utilized to enable a correct localization of the different tracer depots and to allow for repeated determinations of nutrient flow in the same small area The injection needle was kept in place for 20—30 sec to prevent leakage along the needle track Local injections were made either before or after arterial occlusion

In most experiments tracer elimination curves were inscribed during subsequent occlusions of the main trunk of the left anterior artery and of one or two of its main side branches In almost all cases determinations of normal myocardial blood flow (MBF) were performed before and between the occlusion periods The occlusions were usually maintained for 5 minutes or more and at least four minutes intervened between the different occlusion periods

At the conclusion of each experiment and with the heart still beating different dyes were injected through the coronary catheter to stain separately the muscle portions supplied by the respective arteries that had been occluded during the experiment These portions were cut out and weighed both separately and together with the rest of the left ventricle

Results

I Clearance of Kr^{85} and Xe^{133} after close arterial or intramyocardial injection during unrestricted coronary blood flow

During the first 5—15 seconds most clearance curves obtained after close coronary administration of Kr^{85} or Xe^{133} demonstrated an initial phase of a slightly more rapid decay of activity than in the subsequent phase where the curve over a large section displayed a constant disappearance rate In the semilogarithmic plottings of the curves the decay of counting rates usually started to deviate from the monoexponential character when 5—20 per cent

of the peak activity remained to be cleared Mean blood flow (MBF) is calculated from the clearance constant which is derived from the steady main phase of the curve

The initial distortion of the monoexponential decay curve may be considered as an injection artifact due to amongst other things the rapid emptying of the traced blood in the arterial reservoir (HÄGGENDAL NILSSON and NORBACK 1965) This short phase appears to be without importance to the correct evaluation of the main section of the curve Disturbances of the tails of the curves commented on in the first report (JOHANSSON LINDER and SEEMAN 1964) may to a slight extent be due to some recirculation of the isotope which is not eliminated in badly ventilated lung sections Further a delayed clearance from connective and fatty tissues in the ventricular wall and along the coronary vessels may be of special importance for the establishment of the tail of the clearance curve Moreover it is possible that some distortion may be caused by *radiation from tracer amounts already cleared from the myocardium* via the coronary veins These amounts may affect the detector as they pass with the blood stream through the right atrium and ventricle to the lungs via the pulmonary artery

In order to explore the importance of this latter source of disturbance disappearance curves were inscribed after isotope injections on the venous side of the circulation One set of curves shown in Fig 1 were obtained after consecutive injections of identical volumes of a Kr^{85} solution through

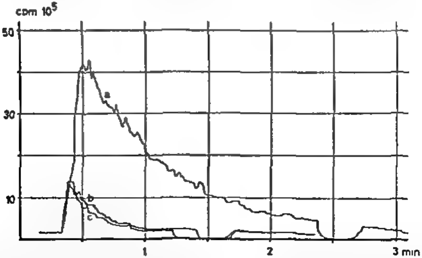

Fig 1 Disappearance curves inscribed after a) close-coronary injection of Kr^{85} b) injection of same tracer amount into the right atrium and c) into the right main pulmonary artery branch All catheters were of same length and lumen size and the injections were made during equal time intervals

either the coronary catheter or catheters placed in the right atrium and the right pulmonary artery branch The injections were made at equal time intervals and stable heart rate and arterial blood pressure prevailed during this whole period The injections of Kr^{85} into the right atrium and pulmonary artery effected almost identical maximal counting rates both corresponding to slightly less than one fourth of the peak activity recorded in the myocardial clearance curve Moreover the activity induced by these injections into the right atrium or pulmonary artery showed such a rapid decay that the isotope concentrations were already close to background level within a minute after the initial concentration rise The elimination of tracer injected into the coronary sinus was studied on a later occasion Except for a peak activity of about 50 per cent of the maximal myocardial concentration this curve differed in no respect from those where the tracer had been injected directly into the right atrium or pulmonary artery displaying the same rapid isotope elimination

Evidently indicator injections direct into the blood passing through the right heart and the pulmonary artery must result in far higher initial concentrations than can ever be obtained by the wash out of the same tracer amount from the myocardium after close coronary injection On the other hand a minor background activity is always present in the blood stream passing through the right heart as long as the isotope in the myocardium is being cleared via the coronary veins However the very marked dilution and the rapid elimination of these tracer amounts passing again close to the detector would seem to reduce effectively their influence on the myocardial clearance curve Similarly the small amounts of isotope recirculating through the lungs should hardly cause any significant distortion of the curve

By contrast slowing of the decay rate at the end of the curve might be at least partly explained by the results of a few recordings extended over 8—10 minutes Half time values of 5 minutes or more were obtained for the final sections of these curves which indicated that there exists at least one slowly circulated tissue component within the myocardium A correction has to be made for the low isotope concentration in this component after a short injection (LASSEN *et al* 1963) The mass of this component probably consisting of connective tissue and/or fat was then estimated at only 5—15 per cent of the total myocardial portion reached by the tracer Subtraction of this slow component from the original curve did not result in a MBF value that differed significantly from the value obtained in the usual way

In order to evaluate the accuracy of the MBF values twelve duplicate determinations were performed in ten dogs with stable heart rate and arterial blood pressure In these 12 pairs of determinations MBF was estimated at the

mean of 123 ml/min × 100 g (range 61–172). The mean difference was not significant (M.D. 3±3, r=0.94). The experimental error is 6.3 per cent of the mean MBF in the whole series. In addition, MBF was determined by tracer injected either close arterially or direct into the myocardium in three experiments. Mean flow values of 129 ml and 126 ml/min × 100 g, respectively, were obtained. The maximal difference between the values in these three pairs of determinations was 12 ml/min × 100 g, i.e. maximally of the order of 10 per cent. Therefore, in the normally perfused myocardium there appears to be a close agreement of the MBF values obtained by either way of administering the tracer. These results are further comparable to those presented below (II) in respect to the degree of correlation between the fast component of the composite curve produced by arterial occlusion after close coronary isotope injection and MBF measured by topical tissue clearance.

II. Clearance of Kr^{85} and Xe^{133} after close arterial or intramyocardial injection during coronary occlusion

a. *Coronary blood flow in the normally perfused myocardium surrounding the ischemic area.* In sixteen experiments a series of repeated occlusions of coronary arterial branches comprising 54 composite curves were performed. The curves were inscribed during two to three occlusions of a single artery. One to three different arteries were occluded in each animal. For the statistical evaluation of the data 30 pairs of values were constructed, thereby pairing the second with the third value in the few instances where three arterial occlusions were performed in a row.

Comparisons were also made concerning the results obtained upon separate occlusions of side branches and the main trunks. From a methodological point of view the clearance curves inscribed during the occlusions of these side branches might be expected to be resolved with less accuracy. Table 1 therefore

Table 1. Comparisons of MBF_{f} at repeated arterial occlusions

Material	Nrs of paired det	1st det ml/min × 100 g Mean ± S.D.	2nd det ml/min × 100 g Mean ± S.D.	Difference ml/min × 100 g Mean ± S.D.	Standard Error	r	P
Total series of occlusions	30	125±24	130±32	5±21	3.91	0.74	>0
Main trunk occlusions	18	117±27	122±37	5±2[illegible]	5.31	0.83	>0
Side branch occlusions	12	135±19	140±27	5±21	5.96	0.[illegible]2	>0

summarizes not only the compiled results in the total series of arterial occlusions including both main trunks and side branches but also the results obtained during occlusions of the main trunks (MT) and of side branches (SB) separately

As seen from the above results the values for MBF derived from the fast component of the clearance curves were reproduced with satisfactory accuracy in the total series of duplicate determinations The standard error of a single determination was estimated at 15 per cent of the mean value for the whole series It appears however that there was a lesser degree of correlation between the paired values in the series of side branch occlusions

Normal myocardial clearance curves were usually inscribed before and after the occlusion periods in the above experiments Such comparative measurements were also obtained in an additional number of experiments This made possible a comparison of the MBF values obtained either from the myocardial clearance curves during totally unrestricted arterial blood supply (MBF_{sep}) or by deducing from the fast components of the composite curves (MBF_{fast}) Further as there was a considerably larger number of determinations available for this comparison the results obtained during occlusions of the smallest of the two side branches in the individual animals could also be evaluated separately The results are summarized in Table 2

Although the comparisons in Table 2 show that the two types of measurements of MBF (MBF_{sep} and MBF_{fast}) did not differ significantly some interesting features were revealed by the results It was noted for example

ble 2 Comparisons of MBF values obtained either at separate determinations or deduced from the fast components of the composite curves

Material	Nrs of paired det	MBF_{sep} Mean ± S D	MBF_{fast} ml/min / 100 g Mean ± S D	Difference Mean ± S D	Standard Error	[illegible]	P
tal series occlu ions	48	129 ± 30	12[illegible] ± 2[illegible]	−2 ± 23	3 [illegible]	0 80	>0 0[illegible]
ain trunk clu ions	16	134 ± 29	119 ± 2[illegible]	−15 ± 30	[illegible] 62	0 36	>0 0
clusions larger side anches	16	123 ± 2[illegible]	128 ± 32	± 11	2 [illegible]9	0 94	>0 0
clusions of all side anches	16	130 ± [illegible]	136 ± 3[illegible]	6 ± 24	5 9	0 [illegible]3	>0 0

that the fast slopes of the curves at main trunk occlusions tended to be slower than those of separate clearance curves. In view of the close agreement between the fast slopes described in the duplicate series in Table 1, the divergent results of the actual comparison might indicate that the unrestricted coronary blood flow decreased during the main trunk occlusions in spite of a nearly unchanged mean blood pressure. The almost lost contractility of a large ischemic wall section may well reduce the over all contraction efficiency of the heart that dilation of the left ventricle occurs and filling pressure is raised considerably. If so, these changes may tend to reduce coronary blood flow, at least to some extent.

The results of the other comparisons in Table 2, where ventricular contractility was less affected because only small branches were occluded, may not only support this last assumption but also justify the conclusion that the fast component of the composite curve reflects correctly the flow rate in the muscle region adjacent to the ischemic portion. As mentioned before, the analysis of the composite curves obtained during occlusions of the smallest side branches, however, being less accurate, explains the lesser degree of correlation between the two types of flow values.

Additional evidence, indicating that the unrestricted myocardial blood flow is on the whole adequately measured by the fast slope of the composite curve, was obtained in a series of comparative local flow measurements. In Table 3 a number of determinations of MBF derived from the fast phase of the composite curve (MBF_{fast}) is compared with an equal number of flow values deduced from the clearance of a tracer depot injected directly into the myocardium just outside the ischemic region (MBF_{local}).

Local clearance curves, reflecting blood flow at the very border of the ischemic muscle portions, were always two phasic. The values for MBF_{local} in Table 3 were therefore derived from the fast components of these curves deduced in the same manner as the fast component of the composite curve obtained upon intraarterial injection. With few exceptions both fast components resulted in closely similar MBF values, as seen from Table 3.

Table 3 Comparisons of MBF values obtained either by the fast components of composite curves or by local clearance curves

Nrs of determinations	MBF_{fast} Mean ± S D	MBF_{local} ml/min × 100 g Mean ± S D	Difference Mean ± S D	Standard Error	r	P
5	112 ± 24	114 ± 2	2 ± 10	4.37	0.9	> 0.0
9	114 ± 21	115 ± [illegible]6	1 ± 19	6.65	[illegible] 0	> 0.05

Based upon the results of the above comparisons it may be concluded that blood flow in the normally perfused myocardium adjacent to an acutely ischemic muscle region is estimated with satisfactory accuracy, both by intramuscular tracer administration and by the fast component of the composite clearance curve obtained by intraarterial tracer administration preceding the arterial occlusion

b Collateral coronary blood flow in the ischemic region The important question as to how the slow component of the composite clearance curve is related to the actual blood flow in the ischemic muscle region (CCBF) is considered to be fairly well answered by the results in the experiments reported in the first paper (JOHANSSON, LINDER and SEEMA 1964) It was for example, here shown that isotope administered distally to a preceeding arterial occlusion was cleared at almost the same rate (CCBF in Table 4) as when injected before the onset of occlusion (CCBF $_{slow}$ in Table 4) A similar experimental procedure was used in two experiments of the present series The results of these three experiments comprising together five measurements of CCBF by either technique are shown in Table 4

It is seen that there was a very close correlation of the values by the two techniques in this small series of measurements As was mentioned in the paper referred to earlier the clearance curves obtained when arterial occlusion preceded the tracer injection described a monoexponential decay of radio activity after about one minute subsequent to the completed injection when a slightly more rapid clearance rate was shown On one occasion however there was a large rapid compartment which was cleared at the same fast rate as a normally perfused muscle region This finding could of course indicate that the artery had not been sufficiently tightened to prevent leakage of the indicator into a proximal muscle portion On the other hand it is also possible that a forceful intraarterial injection may have reversed the flow in large sized collaterals thus distributing the tracer to areas outside the ischemic section

Table 4 Comparative measurements of CCBF by close coronary administration of tracer made either before or after occlusion and distal to the occlusion site

Vessel terminations	CCBF Tracer before occl Mean ± SD	CCBF Tracer after occl Mean ± SD	Difference Mean ± SD	Standard Error	r	P
	ml/min/100 g					
	11.9 ± 4.3	12.6 ± 3.3	0.7 ± 1.3	0.8	0.98	>0.0

Table 5 Comparisons of CCBF values at repeated arterial occlusions

Material	Nrs of paired de termination	1st determination	2nd determination	Difference	Standard	
		ml/min × 100 g				
		Mean ± S D	Mean ± S D	Mean ± S D	Error	r
Total series	3[illegible]	11 9 ± 4 3	1[illegible] [illegible] ± 4 [illegible]	0 [illegible] ± 1 [illegible]	0 [illegible]	0 9[illegible] >
Main trunk occlusion	17	1[illegible] 6 ± [illegible] [illegible]	1[illegible] [illegible] ± 4 9	−0 [illegible] ± 1 1	0 [illegible]6	0 93 >
Side branch occlusions	15	11 [illegible] ± 3 [illegible]	11 [illegible] ± 3 9	0 [illegible] ± 1	0 4[illegible]	0 90 >

The validity of the measurements of CCBF by the flow component of the ordinary composite clearance curve after intraarterial administration of tracer was studied in the series of repeated arterial occlusions already mentioned under II a Altogether 56 arterial occlusions were performed in eighteen experiments The corresponding data for CCBF were grouped in the same way as described for MBF_{fa}, in Table 1 and a total of 32 pairs of CCBF values are shown in Table 5 As in Table 1 both the total series of CCBF determinations and those obtained during main trunk occlusions (MT) and occlusions of the side branches (SB) respectively are given and evaluated separately to disclose any appreciable difference in accuracy As judged from the results in the total series the values for CCBF were reproduced with an accuracy comparable to the values for MBF_{fa}, of the same clearance curves shown in Table 1 The standard error of a single determination of CCBF is 6 3 per cent of the mean value in the total series The mean differences in the two separate series (MT and SB) although not being significant in the individual ones indicated a slightly larger error at occlusions of the two side branches

To evaluate the measurements of CCBF by the composite curves further such curves were compared with those describing the clearance of local tracer depots in 12 experiments The local intramuscular injections into the ischemic area were made at the various locations denoted in Fig 2 and the entire material is shown in Table 6 The tracer was deposited either immediately before or after the arterial occlusions Also included in these local flow determinations are the results obtained in one additional experiment where CCBF was determined by recording the beta clearance from marginal sections of the ischemic myocardial region In this instance the decay of activity was monitored by a GM end window tube shielded with a centrally perforated aluminum sheet (D 0 5 cm)

Fig 2 Locations of intramyocardial tracer depots in 12 experiments and positions of GM detectors in one experiment From left to right during occlusions of main trunk right side branches and left side branches *Arrows denote the assumed central areas of the respective ischemic muscle regions* Tracer injections into same small areas were repeated in several experiments

As illustrated in Fig 2 several tracer depots for measuring CCBF were located exactly on the boundary line between the ischemic and the normally perfused heart muscle These isotope injections were guided by the usually very distinct tissue cyanosis starting at the border of the ischemic region As mentioned earlier in section II.a these decay curves always described a composite tissue clearance quite similar to the clearance of intraarterially administered isotope followed by a period of arterial occlusion and also to the blood flow values obtained Thus the fast phase of the local curve was interpreted as describing blood flow within the normally perfused muscle tissue closely adjacent to the ischemic region which blood flow was evidently represented by the slow phase Typical local clearance curves are shown in Fig 3 Such flow values derived from 10 two phasic local clearance curves and representing CCBF in the ischemic region are compared in Table 7 with the values for CCBF calculated from the slow components of composite clearance curves obtained after intraarterial injections (Table 7)

A somewhat larger number of local clearance curves were inscribed after isotope injections some 5 mm inside the cyanotic border of the ischemic region These curves demonstrated monoexponential wash out processes and tended to result in lower values for CCBF (Table 7) as compared with the values obtained by the slow component of the composite curve where the tracer was placed just at the border (Table 7)

When the tracer was injected into central parts of the ischemic muscle portion the tracer depots were in several instances cleared at definitely reduced rates (Table 7) The mean magnitude of CCBF in the central areas of the

Isotope injected into	Local clearance curves: Nrs of det	Heart rate Mean±S D	MABP mm Hg Mean±S D	CCBF ml/min × 100 g Mean±S D	Fast comp Mean±S D	Ordinary composite curves: Nrs of det	Heart rate Mean±S D	MABP mm Hg Mean±S D	CCBF ml/min × 100 g Mean±S D	MBF_{fast} Mean±S D	Ischemic muscle mass: Rel % Mean±S D	Weight Mean±S D
Main trunk occlusions												
borderline tissue	6	15[illegible]±14	1[illegible]7± [illegible]	16 5±6 6	1[illegible]1±44	4	16[illegible]±10	130± 4	9 1±3	134± 0	50±12	1[illegible]±7
marginal tissue (monophasic)	8	1[illegible]6±1[illegible]	109±24	8 1±4 0	–	6	164± 8	115±[illegible]3	9 7±4 4	1[illegible]1±[illegible]6	[illegible]4±11	1[illegible]±3
central tissue	16	159±17	113±24	4 ±3 8	–	10	1[illegible]8±13	117±[illegible]	12 0±8 5	1 0±33	51±1[illegible]	18±5
Right branch occlusions												
borderline tissue	3	1[illegible]0±10	110±3	11 4±4 4	89±28	3	179±10	113±0	1[illegible] 1±4	101±41	40±4	16±4
marginal tissue (monophasic)	8	173±17	104±17	8 7±3 6	–	5	17 ± 9	117± 7	14 7±7 8	102±[illegible]7	42± 6	14±4
central tissue	1 1	160 166	106 112	1 6 2	– –	1	168	106	7 1	60	36	10
Left branch occlusions												
borderline tissue	1	192	1[illegible]4	9 1	130	1	193	1[illegible]2	17 0	15[illegible]	43	8
marginal tissue (monophasic)	1	163	85	14 4	–	1	[illegible]	88	9	114	[illegible]3	8
central tissue	1 1	184 183	1[illegible]9 117	5 4 14 5	– –	1	180	111	14 8	71	39	11

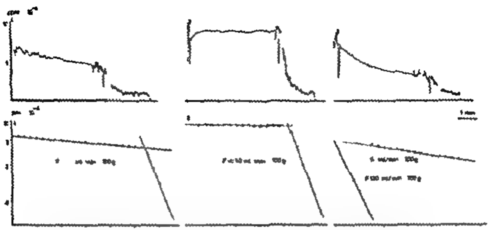

Fig 3 *Above* Wash out curves after local Xe[133] deposits into a) marginal zone b) central area and c) borderline tissue of ischemic muscle region resulting from main trunk occlusions in one experiment *Below* Semilogarithmic plottings of the curves Curves a and b describe almost monoexponential decay of counting rates while c is biphasic Rapid wash out rates after release of occlusion were not appreciably different

ischemic regions thus amounted to approximately half the value of that at the marginal zones In other words the above findings during local flow measurements strongly argued for the existence of a somewhat inhomogenous blood flow within the ischemic muscle portion

Table 7 Comparisons of CCBF values obtained either by the slow component of composite curves or by the clearance rates of local tracer deposits into same ischemic muscle regions

Isotope injected into	Nrs of determinations	$CCBF_{loc}$ ml min × 100 g Mean ± S D	Nrs of determinations	$CCBF_{1}$ ml min 100 g Mean ± S D	Difference of means ± S D	P
borderline tissue (biphasic curves)	10	14 ±6 1	8	11 ± 4	3 0 ± 1	>0 05
borderline tissue (monophasic curves)	1	8 ± 4 3	1	11	3 1 ± 1	>0 05
tissue in total marginal zone	[illegible]	10 8 ± 4 9[1])	20	11 3	[illegible] ± 4	>0 0
central tissue zone	0	4 8 4 0[1])	13	12 3 ± 7 6	7 5 ± 4 0	>0 0

[1]) Diff of mean values [illegible] 1 [illegible] 1 < 0 0

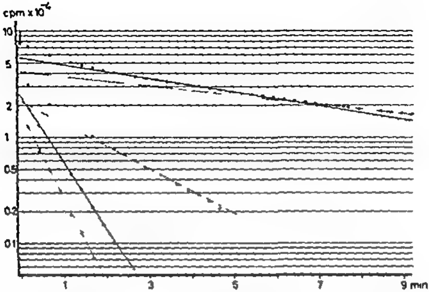

Fig 4 Semilogarithmic plotting of a composite curve obtained after close arterial Xe[133] injection and during 9 minutes occlusion period Compartmental analysis based on final curve section results in three components the slopes denoted by hatched lines Solution of curve obtained during first five minutes of occlusion results in two well defined components denoted by two continuous slope lines The fast slope of latter curve describes a rate constant almost identical with that obtained from a preceeding monoexponential curve The fast slope deduced from 9 min curve shows an appreciably higher rate constant See further discussion in the text

A similar interpretation is suggested by the slightly diminished clearance rate observed towards the end of some composite curves inscribed during prolonged arterial occlusions This is illustrated in Fig 4 by the semilogarithmic plot of a composite clearance curve inscribed during an occlusion period of more than 9 min duration If the solution of this curve is confined to the usual 5 min occlusion period it results in only two distinct components with half time values of 4 64 and 0 48 min respectively The latter value agreed closely to the value of 0 50 min of a preceeding monoexponential curve When on the other hand the analysis is based on the entire 9 min period three separate slopes are obtained one slow one intermediate and one fast component Half time values for the respective slopes were 8 95 1 18 and 0 38 It is also seen that the mass of muscle cleared at the intermediate rate constituted about half that of the slowest component as estimated by the respective zero time ordinate values When added together these two components correspond to a mass which is some ten per cent larger than the tissue mass of the single slow component deduced from the actual composite

curve when recorded during the first five minutes of occlusion Actua it may be assumed that there existed within the ischemic region a wh series of components becoming gradually slower and suggesting the cleara of more central areas of this region The result of such an analysis of the sl components of the curve would presumably be an artificial organization zones of gradual flow shifts ending up with two zones of average flows general however it may be concluded that the rate constant of the sl component of the two-compartment curve reflects in approximate figu the average flow rate within the main portion of the ischemic muscle regio

Fig 5 taken from one experiment where blood flow was measured both close arterial and intramuscular tracer injection illustrates in a simplif way the estimated flow rates around and within an ischemic muscle porti

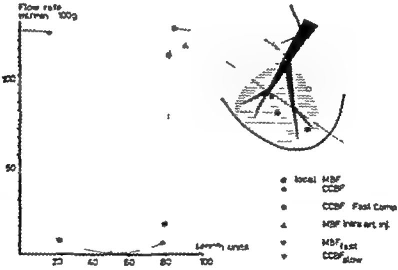

Fig 5 Simplified profile of flow rates around and within the ischemic muscle porti resulting from main trunk occlusion in one experiment The oblique line in the schemati drawing denotes location and length relationships in arbitrary units assumed for t profile Ischemic muscle weighed 19 g (=17% of LV weight) and as estimated by t resultant component clearance curve comprised 60 per cent of total region into whi tracer was distributed by close-coronary administration. Blood flow was measured both close arterial and intramyocardial injection of Xe 133 whether followed or not by arte occlusion Flow values are given in ml min × 100 g

Xe 133	MBF		CCBF	
	Sep det	Fast comp	Slow comp	Single comp
Close-arterially	116	124	–	
Intramuscular	111			
marg tissue		125	16 6	
marg tissue				6 6
central tissue				1 0

III Reactive hyperemia phase after release of coronary occlusion as measured by close arterially and intramuscularly administered Kr^{85} and Xe^{133}

To obtain satisfactory final wash out sections of the clearance curves the occluded artery must be released while there is still a comparatively high isotope concentration in the ischemic regions because of the usual very rapid wash out rate during reactive hyperemia In most instances peak flow rates of 300—500 ml/min × 100 g are encountered Obviously this final section of the curve should also be inscribed at higher paper speed which was unfortunately not done in a routine way during this study As a consequence the available data are not sufficient for an extended evaluation of the clearance technique for determination of the reactive hyperemic blood flow

The rapid wash out of residual activity within the ischemic region usually starts within two to six seconds after release of the occluded artery It occurs as a monoexponential function at least for the first 15 to 30 sec during which period in most instances the activity has fallen close to background level For this reason it would seem as if the clearance curve relatively adequately measures the peak flow of the reactive hyperemia In a series of 14 duplicate determinations of peak flow the values were reproduced with satisfactory accuracy Peak flow was estimated at a mean of 382 ml/min × 100 g in the whole series The mean difference between the duplicate values was not significant (M D 1±10 r=0 92) The methodological error of 7 per cent of the mean is comparable to the errors obtained for the measurements earlier discussed under I and II

To gain more detail on this aspect of tissue clearance techniques the distribution of reactive hyperemic flow rates within previously ischemic muscle portions was investigated in a series of local flow measurements where the tracer was administered intramuscularly Fig 6 shows the final wash out

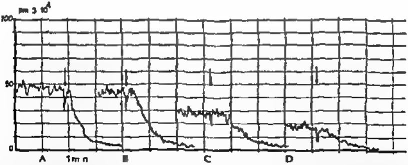

Fig 6 Peak blood flow (PHF) during reactive hyperemia determined by rapid wash out rates of activity upon release of same artery occlusion four times consecutively A close arterially administered Kr^{85} B Xe^{133} depot in centre of ischemic region C Do in marginal tissue zone (monoexponential decay curve) and D Do in the borderline tissue (bi phasic curve) PHF amounted to 299 239 157 and 145 ml/min × 100 g respectively

sections of one composite curve (intraarterial administration of the tracer) and three local clearance curves (intramuscular administration of the tracer) upon release of the same artery which was occluded four times consecutively for the same period of time The three intramuscular injections were placed in the central and marginal areas of the ischemic region and at the very boundary of the non-ischemic portion as judged from the rather distinct line of cyanosis The peak hyperemic flow rates calculated from these four curves were 292 239 157 and 145 ml/min / 100 g respectively thus demonstrating declining values for hyperemic blood flow towards the periphery of the ischemic region Similar measurements were performed in four additional experiments but flow values for the marginal zones of these ischemic regions were not so convincingly lower as in the former experiment which might at least be partly explained by slightly different locations of the intramyocardial depots in the latter series These results show however that the mean hyperemic flow after release of a branch occlusion was significantly lower than the mean flow after release of main trunk occlusions (Table 8) The ischemic area in the latter situation was naturally larger than when only a side branch was occluded and to judge from the results had presumably a lower blood flow in its centre as reported under II b

II Measurement of the relative mass of ischemic myocardium by elimination curves recorded after intraarterial administration of Kr^{85}

The relative size of the ischemic region as a fraction of total tissue mass from which Kr^{85} is cleared after close arterial injection should be reflected in the zero time intercept ordinate of the slow component At the time of

Table 8 Peak flow rates (PHF) during reactive hyperemia measured by the rapid wash-out sections of composite and local clearance curves inscribed after release of arterial occlusion

Release of	Nr of det	PHF locally ml min 100 g Mean ± S D	Nrs of det	PHF tot region ml min × 100 g Mean ± S D	Difference of Means ± S D	P
A Main trunks	11	232 ± [illegible]3	8	210 ± [illegible]8	− 22 ± 30	> 0 05
B Main trunks	13	19[illegible] ± [illegible]	8	213 ± 83	18 ± 4[illegible]	> 0 05
B Side branches	6	1[illegible] ± 49	4	130 ± [illegible]	− 2[illegible] ± 34	> 0 05
[illegible] C		[illegible] ± 2[illegible] [1])		80 ± 44		

A = intramyocardial injection into the central area
B = intramyocardial injection into marginal tissue
[1]) P < 0 0

occlusion this latter value $A_{0,slow}$ may be expressed in per cent of total activity $A_{0,slow}+A_{0,f}$ to give a percentage figure for the relative size of the ischemic portion. This calculation is based on the fact that the initial Kr^{85} concentration is equal in both compartments as pointed out in previous papers (JOHANSSON, LINDER and SEEMAN 1964, 1965). The relative masses of the two myocardial compartments eliminating Kr^{85} were given as a quotient fast/slow (F/S) in the earlier studies. These F/S values were clearly related to the size of the obstructed vascular regions but they were reproduced only with fair accuracy in a series of duplicate determinations (JOHANSSON, LINDER and SEEMAN 1965). For such reasons it appeared that changes in the mass of ischemic muscle might be difficult to detect if induced by altered hemodynamic parameters by pharmacological means or by surgical intervention.

Experiments in the present series suggest that these size measurements may be repeated with improved accuracy if particular attention is paid to standardization of injection technique, careful marking of the coronary occlusion time, duration of recording before occlusion is released, etc. The results of duplicate determinations of the relative size of the ischemic regions performed with such precautions in 16 experiments are reported in Fig. 7. The relative sizes are given in percentage values $100 \cdot A_{0,slow}/A_{0,slow}+A_{0}$ as indicated above. A total of 58 determinations were done at occlusions of

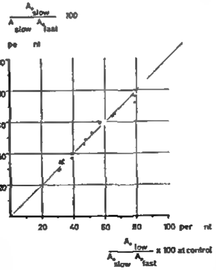

Fig. 7. Results of duplicate determinations of the relative size of the ischemic region produced by occlusions of 22 different arteries in 16 experiments. The mean difference between the 30 paired estimates was not significant. r=0.97. Line of identity is shown.

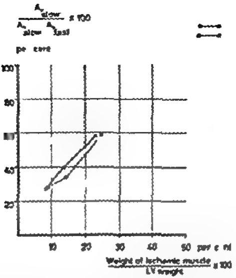

Fig 8 Relationships between mean weight estimates and mean relative size estimates for three different ischemic regions in each of eight experiments (   ) and for two to three regions in twenty four animals (————) Total results in these series of experiments are reported in Table 9 Relevant data for this figure are as follows

Nrs of arteries	Small side branches Weight %	Rel size %	Nrs of arteries	Large side branches Weight %	Rel size %	Nrs of arteries	Main trunks Weight %	Rel size %
8	5 9	26 6	8	14 2	33 3	8	24 8	68 1
15	8 6	27 0	2[illegible]	13 4	30 4	19	2[illegible]	57 [illegible]

22 different arteries two or three times consecutively The mean difference between the values in the 30 pairs was not significant (M D −0 33±2 10 I 0 0 r=0 9")

The correlation between the size of the ischemic regions determined in this way and the actual weight of the myocardial portions supplied by the occluded arteries was also studied The distribution of the coronary arterial branches was mapped out at the end of the experiments on the basis of vascular anatomy and the results of dye injections and the different portions of the myocardium were cut out and weighed Fig 8 illustrates the correlation found between these weights in per cent of total left ventricular weight and the respective percentage values for the relative size of the ischemic portions derived from the clearance curves in two series of experiments Total results in the two series are summarized in Table 9

Table 9 Total data in the experiments reported in Fig. 8

Arterial size	Nrs of arteries	Heart rate Mean ± S.D.	MABP mm Hg Mean ± S.D.	Mass of ischemic muscle Weight g Mean ± S.D.	Rel. mass % Mean ± S.D.	CCBF ml/min × 100 g Mean ± S.D.	MBF_{isch} Mean ± S.D.	MBF_{sep} Mean ± S.D.	r_{MBF}
Small side branches	8	1[illegible]0 ± 20	137 ± [illegible]2	8.9 ± 2.6	20.6 ± 5.3	2[illegible].0 ± 8.6	16[illegible] ± [illegible]0	161 ± [illegible]	0.96
Large side branches	8	171 ± 30	138 ± 21	14.3 ± 5.4	33.4 ± 7.4	19.0 ± 7.0	145 ± 34	14[illegible] ± 36	0.83
Main trunks	8	173 ± [illegible]0	13[illegible] ± [illegible]	[illegible]4.6 ± 5.0	[illegible]1 ± 10.9	9.[illegible] ± 2.[illegible]	118 ± 21	141 ± 39	0.48
Mixed series									
Small side branches	1[illegible]	163 ± 21	1[illegible]3 ± [illegible]0	8.6 ± [illegible].4	[illegible]0 ± 5.[illegible]	0.0 ± 7.0	1[illegible] ± 38	145 ± 43	0.04
Large side branches	[illegible]	161 ± [illegible]6	1[illegible] ± [illegible]5	13.4 ± 4.3	39.[illegible] ± 11.4	16.[illegible] ± [illegible].6	137 ± 3[illegible]	135 ± 33	0.8[illegible]
Main trunks	10	164 ± 27	1[illegible]0 ± 25	[illegible]6 ± 5.0	27.5 ± 14.0	9.9 ± [illegible].1	118 ± [illegible]0	133 ± 37	0.65

Discussion

The purpose of the present report was to analyse in more detail the Kr^{85} and Xe^{133} clearance method earlier developed mainly for measuring collateral blood flow to the ischemic heart muscle of dogs (JOHANSSON, LINDER and SEEMAN 1964, 1965). This flow measurement is based on the composite clearance of an intraarterially administered radioactive inert gas both from the ischemic muscle portion and from adjacent parts of the normally perfused myocardium. It permits measurements of average blood flow both in the ischemic region and in the surrounding normally perfused muscle region and allows, in addition, an approximate estimation of the common masses of the two portions. Further, upon release of the temporary artery occlusion the residual wash-out process appears to reflect the peak flow rate during the period of reactive hyperemia within the previously ischemic muscle section.

In the present study attempts have been made to analyse to what extent these flow measurements, based on an analysis of such composite curves, are reproducible and to what extent they correspond to topic flow measurements where the tracer is administered by intramuscular injection.

With regard to the blood flow rate in the normally perfused myocardium these comparative studies show, on the whole, a good correlation, as occurs when the tracer elimination reflects flow in muscle areas which surround an ischemic tissue region. In general it therefore seems safe to conclude that in the steady state situation Kr^{85} and Xe^{133} clearance curves reflect with satisfactory exactness the flow in normally perfused myocardial sections. Similar results were also reported recently (SULLIVAN *et al.* 1965). Moreover, this agrees with results of other investigators, where the clearance method was compared with more direct methods for determining coronary blood flow (HERD *et al.* 1962, ROSS *et al.* 1964).

It is true, however, that the measurements also revealed the presence of a slowly perfused tissue compartment in the heart, probably corresponding mainly to its connective and fatty tissues. This compartment is, on the other hand, normally so small and so slowly perfused that it will hardly distort the fast component significantly, reflecting the mean blood flow to the heart muscle.

As another potential error it should here also be mentioned that a variable part of the right ventricular muscle was included in the tissue mass from which clearance of the tracer was recorded in at least some of the present experiments. This is because the left descending artery sends off small branches to supply a narrow band of the adjacent right myocardium (BLAIR 1961).

Quite large variations were seen both in the number and size of such branches situated below the coronary catheter and occlusion sites and of course also in the mass of right muscle supplied by them. These portions were not weighed separately, but experience shows that they generally do not make up more than 10—20 per cent of the total ischemic area.

The main reason for discussing this small right ventricular compartment in this connection is that it normally shows a lower blood flow per unit weight than the left myocardium (e.g. ECKENHOFF, HAFKENSCHIEL and LANDMESSER 1947, GREGG *et al.* 1951, ROSS *et al.* 1964). According to these studies the blood supply to the right ventricle is only about half of that to the left ventricle during normal resting conditions. These earlier results could incidently be confirmed in one of the present experiments, wherein blood flow was repeatedly determined by the simultaneous clearance of beta radiation from small superficial areas of the right ventricle (47 ml/min/100 g) and the left ventricle (100 ml/min/100 g). It is then probable that the mass of right muscle involved in the wash out process would, if sufficiently large, be reflected as a second rapid component in the clearance curve. However, it did not show up in the above mentioned curves inscribed during extended periods of time. It is likely that in many experiments the mass of right muscle was simply too small and its flow rate too close to that of the left muscle to show up clearly in the composite curves. In some of the experiments, however, where the right ventricular compartment was larger, it probably introduced a small error in the flow value determined for the left myocardium.

Interest was, however, mainly focused on the measurements of collateral coronary blood flow in a myocardial area distally to a coronary occlusion, and such measurements were also performed with the two named techniques for tracer administration. These experiments, too, indicate that there is, on the whole, a good correlation between the flow rate deduced from the second slow component of the composite curve after intraarterial tracer injection and that deduced from the wash out curve of a tracer depot in the ischemic region. It is, however, also obvious that the second slow component of the composite curve only reflects an approximate average of the collateral blood flow within the ischemic area, and that this flow in reality tends to decline gradually towards the centre of the area. Such a view is supported by several observations. If the recording of the tracer wash out is continued for periods up to 9—10 minutes or more, the slow component of the composite curve tends to exhibit at least two slow phases. It is indeed likely that these merely conceal a whole family of such slow phases, but it is rarely possible to solve for more than three or four components in a multicomponent curve with a reasonably good chance that they will describe real flow events. However

the clearance from topical applications of tracer depots localized to the borderline to the outer and the central zones of the ischemic area suggests that the collateral inflow to this area is abruptly and strongly reduced quite close to the borderline section to become more gradually reduced towards its centre Also the fact that the reactive hyperemia appears to be most intense in central parts of a previously ischemic area speaks in favour of such a graded heterogeneity of the collateral blood supply

Moreover as mentioned above the flow in the small right heart compartment is normally different from that of the left ventricle and will of course also affect the slow component of the composite clearance curve once ischemia is induced Such a factor too may contribute to an uneven flow distribution within the ischemic area It is not unlikely however that the collateral supply to the ischemic right myocardium is relatively more satisfactory than that to the left myocardial portion It will for example be even less interfered with during systolic contraction than the normal blood supply Further the higher density of collateral vessels in the septum and the apex region (e g BELLMAN and FRANK 1958) may also have contributed to a better collateral supply of the right regions If anything such factors would tend to make the collateral flow in the right and left regions more equal than during normal pressure perfusion when the right heart section can hardly be revealed in the clearance curve

In any case the rapid decline of the flow at the border of the ischemic region and the existence of some heterogeneity of the collateral flow in the main ischemic portion will no doubt render the composite curve less valid from the strictly theoretical point of view In a correct measuring of the blood flow by a composite clearance process analysing its different components it is essential that the recording is continued until the slowest component is clearly reflected The errors to be ascribed to such theoretical inadequacies of the composite curve analysis are fairly small judging from the combined experimental evidence They hardly invalidate the two component model used in describing with fair accuracy the average flow conditions within the two muscle portions There is after all generally good agreement between the slow phase of the composite curve and the integrated values obtained by the topical flow measurements On the basis of these results and the accuracy of the fast component in reflecting the unrestricted blood flow rate it is justifiable to consider the slow component as a representative exponent of a family of slow blood flow rates existing in the main portion of an ischemic region

The theoretical inadequacies of the composite curve mainly appear to be limited to its deficiency in revealing separately the slowest flow rates perfusing

the central part of the ischemic portion. Judging from experiments where the retrograde flow and pressure are measured the collateral flow seems to reach a critically low level, it evidently being driven by a very low pressure head with a low transmural pressure distending the vessels (e.g. GREGG 1950). It can therefore be expected that even minor shifts in the local pressure head, the diastolic ventricular pressure and the relationship between systole and diastole produce considerable changes in this flow. The vessels in central parts of the ischemic zone are largely maximally dilated and at low pressures the pressure flow relationships for dilated vessels are such as to give great changes of flow for rather small changes in pressure (e.g. FOLKOW and LÖFVING 1956).

As a steady state in all the parameters mentioned above are hardly ever to be expected over periods of say 10—15 minutes it follows that the slow blood flow in these central parts of an ischemic myocardial area probably changes considerably from moment to moment. If so it is in any case impossible to get a uniform slope representing this flow, even if the theoretical conditions for revealing such a slope had been ideal. In other words, the biological variables *per se* are in all probability so considerable in this very situation that little is to be gained by further improving the analysis of the composite curve. To obtain more detailed information it might then be more profitable to combine the two-compartment analysis with topical flow recordings based on local intramuscular injections as was the case in the present study.

The practical use of clearance techniques for measuring blood flow during reactive hyperemia is then more debatable than when they are utilized for the determinations of myocardial blood flow under steady state conditions. According to earlier investigators blood flow during myocardial reactive hyperemia reaches a maximum within a few seconds after release of arterial occlusion, continues at this peak rate for a short period and then slowly returns to the control level (e.g. COFFMAN and GREGG 1960, DRISCOL, MOIR and ECKSTEIN 1964). Thus blood flow changes almost continuously during the period of reactive hyperemia as does the rate of tissue clearance of radioactive tracers. However, the longer the period of ischemia the longer the period of peak hyperemia flow in most tissues. It was also demonstrated in an earlier report (JOHANSSON, LINDER and SEEMAN 1964) and is further confirmed by the present results, that the initial extremely rapid wash-out phase describes a fairly monoexponential decay of activity from 4 to 8 seconds after release of arterial occlusion until 30 or 45 seconds later. The counting rates after this period of time are usually close to the background, indicating that there hardly exist any limiting diffusion distances with respect to the capillary network

of the myocardium. However, it is also clear that the wash out curves can be used for measuring peak flow rate and only when the latter stays fairly constant for some period of time, as is the case after prolonged periods of ischemia. The subsequent gradual decline in the hyperemia can not be correctly judged from the wash out and furthermore, the peak flow will have washed out practically all tracer present. This is in contrast to the situation during reactive hyperemia in skeletal muscles (LASSEN, LINDBJERG and MUNCK, 1964; LASSEN, LINDBJERG and DAHN, 1965), probably due mainly to the fact that peak hyperemia flow in heart muscle is almost ten times as high as in skeletal muscle.

The validity of the composite curve in most steady state conditions is evident when used in estimating the size of the ischemic muscle portion. Provided certain precautions are taken, such estimations agree fairly well with the directly measured weight of the ischemic region, which becomes easily visible because of the usually distinct borders and obvious cyanosis. Clearly, estimation of the ischemic mass by the composite curve requires that the two main components of the curve are distinctly revealed, i.e., the rapid component must be cleared completely. The slower the flow in the well perfused areas and the smaller the ischemic section, the greater the practical difficulties in obtaining reliable measurements.

The extension of the ischemic zone can also be checked by local flow measurements. As mentioned earlier, when compared with the normally perfused myocardium, such measurements demonstrated a marked drop in blood flow already a few mm inside the visible border of ischemia. Therefore, at least when larger tissue masses are made ischemic, the marginal zone appears to comprise only a fairly minor fraction, but this fraction naturally increases as the ischemic region becomes smaller.

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EFFECTS OF HEART RATE AND ARTERIAL BLOOD PRESSURE ON CORONARY COLLATERAL BLOOD FLOW IN DOGS

By

BÖRJE JOHANSSON, ERLAND LINDER AND TORSTEN SEEMAN[1]

ABSTRACT

BÖRJE JOHANSSON, ERLAND LINDER and TORSTEN SEEMAN. *Effects of heart rate and arterial blood pressure on coronary collateral blood flow in dogs.* Acta physiol. scand. 1966. *68*. Suppl. 272. 33—46.

The Krypton[85] clearance technique was used for studying the effects of induced changes in heart rate and arterial blood pressure on the collateral blood flow of the myocardium during acute coronary occlusions in dogs. Collateral blood flow was little affected by variations in heart rate although a slight improvement of flow was seen at frequencies between 90 and 130 beats/min compared to higher frequencies. Arterial blood pressure is an important determinant of coronary collateral blood flow in the non failing heart, but the pressure flow relationships indicate a passive behaviour of the collateral vessels. Ventricular dilatation with elongation of the collateral vessels and increased intramural pressure seems to impede collateral flow. The size of the ischemic region produced by the coronary occlusion is not significantly altered by changes in heart rate or blood pressure.

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Introduction

Our present knowledge of the collateral circulation in heart muscle is incomplete in several important respects despite major advances in recent years. To a large extent this is due to technical difficulties and inadequate methodology. Measurements of retrograde flow from a coronary artery distal to an acute or chronic occlusion have been used as an index of collateral blood flow (ANREP and HAUSLER 1928, ANREP, BLALOCK and HAMMOUDA 1929, MAUTZ and GREGG 1937). The available information concerning the determinants of coronary collateral blood flow (CCBF) is derived mainly from such studies. It has been shown that the magnitude of retrograde flow is closely dependent on actual perfusion pressure (mean arterial blood pressure), on hematocrit and whole blood viscosity, and on the degree of distention or stretch of the myocardium (WIGGERS and GREEN 1936, KATTUS and GREGG 1959). Conversely, wide variations in heart rate did not affect retrograde flow to any significant extent in the study by KATTUS and GREGG. ECKSTEIN (1954) assumed that retrograde flow overestimates collateral blood flow by some 10 per cent and this opinion was later shared by KATTUS and GREGG (1959). On the other hand, recent studies of the myocardial uptake of Rb^{86} (LEVY, IMPERIAL and ZIESKE 1961) have suggested rather the opposite, indicating that the ischemic myocardium receives certain amounts of collateral blood which are not measurable with the back flow technique. Methods based on the clearance of radioactive tracers measuring capillary myocardial blood flow would seem to offer improved opportunities for the study of factors determining CCBF.

The Kr^{85} clearance technique has been found to be a practicable method for determination of CCBF and it permits, in addition, an estimation of the relative size of the respective ischemic muscle region (JOHANSSON, LINDER and SEEMAN 1964, 1965). In a recent evaluative study of the method (LINDER 1966) it was shown that reliable determinations can be made under varying cardiovascular conditions and with different types of occlusion. Hence it was considered to be of interest to apply this technique in a study of the effects of heart rate and arterial blood pressure not only on coronary collateral blood flow but possibly also on the size of the ischemic region.

Methods

The results to be reported were obtained in experiments on seventeen dogs (17 to 38 kg). The fasting animals were anesthetized with pentobarbital intravenously, curarized, intubated and artificially ventilated with oxygen and nitrous oxide in the Engström respirator without rebreathing.

The heart was exposed through a left or bilateral thoracotomy and the widely incised pericardium was sutured to the thoracic wall. In most animals two stimulation electrodes were sutured to the right ventricle followed by injection of small quantities of formaline or absolute alcohol into the ventricular septum in order to produce total A—V block. Pacing of the heart was then done with a Grass stimulator (impulse duration 1 msec at 3—5 V).

Short sections of the main branches and or the trunk of the left anterior descending artery were dissected for later occlusion with snares. A polyethylene catheter was inserted either into the central end of a small side branch or sutured directly into the artery a short distance proximal or distal to the occlusion levels. In experiments with distal catheters retrograde pressure was recorded during the occlusion periods. ECG and intraarterial blood pressure were recorded continuously throughout the experiments except in those few cases where pressures from multiple sites in the central circulation were measured. In these experiments it was necessary to alternate between two or three pressures on the same recording unit. All pressures were obtained with transducers of the variable inductance type (Elema) and recorded on a four channel direct writing oscillograph (Mingograph Elema). Mean pressures were obtained by electrical integration. In a few animals multiple determinations of cardiac output were made with the krypton dilution technique of CORNELL, BRAUNWALD and BROCKENBROUGH (1961). Most animals in this study were heparinized (1.0—2 mg/kg) to prevent clotting.

For each measurement of flow 0.5—2 ml of a saline solution of Kr^{85} was injected into the coronary catheter over a period of about 5 sec. An external scintillation detector placed above the anterior wall of the left ventricle monitored the decay of radioactivity which was then converted and recorded on a potentiometer writer. The elimination curve inscribed during arterial occlusion was evaluated according to the method described in closer detail in the previous reports (JOHANSSON, LINDER and SEEMAN 1964, 1965; LINDER 1966). In principle the slope of the slow component obtained in the semilogarithmic plotting of the curve was used in calculating the coronary collateral blood flow supplied to the ischemic region. The slope of the fast component represents the unrestricted blood flow within the adjacent muscle eliminating the isotope after the injection. To obtain a measure of the relative size of the ischemic muscle region the activity value of the extrapolated slow component at the moment of arterial occlusion was expressed as a percentage fraction of total activity recorded at this moment $A_{0} = 100/A_{0s}+A_{0f}$ (LINDER 1966).

Variations in heart rate were effected by stepwise increase or decrease of stimulation frequency. In one animal with normal A—V conduction heart

rate varied spontaneously. In all these experiments arterial blood pressure was kept as nearly constant as possible by a pressure bottle system connected to one femoral artery.

Arterial blood pressure was varied by graded bleeding or transfusion of the animals or by pharmacological means in some of them. In the latter case blood pressure was lowered by intravenous infusion of trimethaphan (Arfonad), whereas elevation of pressure was effected by infusion of metaraminol (Aramine) intravenously. In order to suppress ectopic ventricular foci evoked by the latter drug these hearts were paced at comparatively high frequencies.

In either type of experiments no measurements of flow were carried out until steady state conditions had been reached as judged from a stable blood pressure and a constant blood level in the pressure bottle. Repeated determinations of hematocrit in samples of arterial blood showed that this parameter varied insignificantly during the course of the experiments.

Results

Fig. 1 shows the values for coronary collateral blood flow obtained during arterial occlusions at different heart rates in five animals. The results are presented as eleven sets of values each representing individual arterial branches subjected to occlusion. Significant changes in arterial blood pressure could be avoided despite the wide variations in heart rate in all experiments except one. In the latter, where heart rate was increased from 105 to 150 and then to 190 beats/min there was a gradual fall in blood pressure which may have contributed to the fall in CCBF. Fig. 1 shows a general tendency to higher values for CCBF at heart rates below 130 beats/min while higher frequencies seem to be of little influence.

The flow values reported in Fig. 1 represented collateral blood flow within ischemic muscle portions of relative masses ranging from 13 to 78 per cent. To find out whether this relative size of the ischemic muscle resulting from occlusion of a particular artery would depend on the actual heart rate fifteen pairs of estimates have been plotted for comparison in Fig. 2. In this figure the percentage value obtained for an artery occlusion at the lowest heart rate (abscissa) has been paired with one or two values obtained when the same vessel was clamped at higher heart rate (ordinate). The points are seen to scatter on both sides along the line of identity and there is evidently no systematic effect of heart rate on the relative size of the muscle mass subjected to ischemia. The mean difference in this material is closely similar to that found in the series of duplicate determinations reported by LINDER (1966) being less than one per cent and statistically not significant (M.D. −0.3 ±

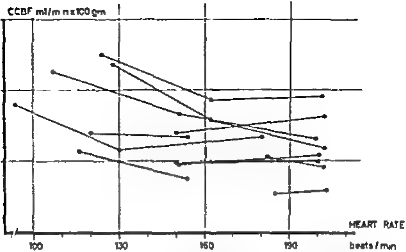

Fig 1 Changes in CCBF during occlusions of eleven arteries at two to three different heart rates Each set denotes the results for a single artery There is a tendency to higher CCBF values at heart rates below 130 beats/min

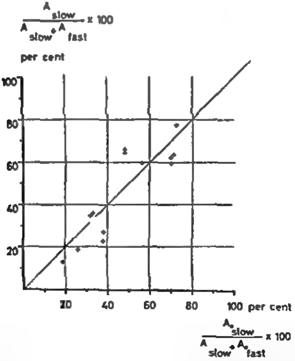

Fig 2 Variations in the relative mass of ischemic muscle resulting from an artery occlusion at different heart rates Estimates at lowest rates have been compared with one or two values at higher rates The results refer to the eleven arteries reported in Fig 1 The mean difference was not significant r=0 88

±2 S.E.). The standard deviation of the difference was 9.8% as compared to 3.0% in the series of duplicate determinations reported by LINDER (1966). This larger deviation in the heart rate series may be due to the fact that the coronary catheter was placed distal to the site of occlusion in two of these experiments and this arrangement has been found to effect larger variations in the estimation of size of ischemic region. Retrograde pressure was recorded at the different heart rates in these two experiments. It was found to rise or remain unchanged despite that CCBF dropped when heart rate was stepped up from the lowest frequency. This would indicate an increasing resistance to flow in the peripheral vascular bed of the ischemic region with increasing heart rate.

To study the effects of actual perfusion pressure on the collateral blood flow within an ischemic muscle region and on the size of this region, coronary artery occlusion was repeated at different levels of mean arterial blood pressure accomplished by bleeding or transfusion of the animals or by pharmacological means. The results to be presented were obtained during 51 arterial occlusions made at two to five mean blood pressure levels in experiments on seventeen dogs. In eight of these experiments two or three different branches were subsequently occluded at the respective pressure levels. Owing to the artificial stimulation of most hearts in this study variations in heart rate were kept within 10 beats/min. Only in the experiments on two dogs with maintained A–V conduction did heart rate differ by up to 49 and 30 beats/min respectively between the determinations compared. The experiments have resulted in 21 sets of pressure flow relationships which are demonstrated in Fig. 3. To provide a general impression of the distribution of the sets of values with regard to the flow resistance the diagram has been divided into four resistance regions by the dashed lines drawn from origin. Collateral flow resistance is here expressed in relative units (CCRU) calculated as mean blood pressure (mm Hg) divided by CCBF (ml/min × 100 g). Coronary collateral blood flow was determined at mean blood pressures ranging from 32 to 213 mm Hg in the total series of arterial occlusions and CCBF varied from 3.8 to 37.8 ml/min × 100 g. The largest difference obtained between the values of CCBF in a single set amounted to 22.6 ml/min × 100 g (33.8 to 11.2) when blood pressure dropped from 135 to 75 mm Hg subsequent to the stopped infusion of aramine. The other extreme was observed in the experiment where CCBF increased from 4.? to 7.8 ml/min × 100 g despite a drop in blood pressure from 197 to 66 mm Hg due to bleeding of the animal. This was the only experiment clearly demonstrating an adverse effect of high arterial blood pressure on CCBF. Left ventricular diastolic pressure incidentally measured in this experiment showed a marked elevation to 32 mm Hg during the period of

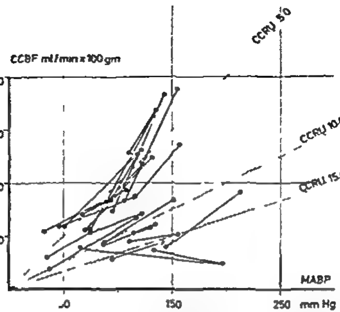

Fig 3 CCBF in relation to mean arterial blood pressure Twenty one pressure flow relationships were obtained during 1 arterial occlusions at two to five blood pressure level. CCRU denotes total collateral flow resistance in relative units (MABP in mm Hg/CCBF in ml min x 100 g)

high arterial blood pressure indicating that severe strain was imposed on the left ventricle The resistance to CCBF during this period reached an exceedingly high value of 42 units

In general the effects on CCBF of different blood pressures irrespective of the manner in which they were achieved indicated only moderate or insignificant changes in flow resistance Thus the mean difference between the resistance values calculated for the highest and the lowest pressure level in each set of values in Fig 3 were not statistically significant (M D −0 2 ± 1 8 S E) The distending effect on the collateral vessels of higher blood pressure appears to be present only at the lower resistance levels as denoted by the sets of values around 5 0 resistance units

As indicated by the results reported above the collateral vessels would seem to function as passively extensible tubes Evidence supporting this view was obtained by simultaneous measurements of aortic blood pressure (MABP) and retrograde pressure (MRBP) in nine arteries occluded during experiments on six animals The CCBF value together with the effective perfusion pressure over the interarterial collateral vessels (MABP—MRBP) and over the peripheral vasculature of the ischemic region (MRBP—right arterial pressure PAP)

make it possible to estimate the flow resistance of the respective vascular sections. Such calculations are based on the assumption that the entire CCBF passes through both these series coupled resistances. RAP has not been recorded regularly in the experiments and MRBP has therefore been taken as the perfusion pressure of the peripheral vasculature.

The pressure flow relationships obtained for the interarterial collateral channels are shown in Fig. 4 and for the peripheral vessels in Fig. 5. It is evident that the former vessels constitute the major part of the total resistance to CCBF. Only twice among the twenty two determinations were the two values of similar magnitude. The resistance of the collaterals was 1.5 to 5 times that of the peripheral vessels in the rest of the material. Thus it appears that CCBF will only rarely and probably to a minor degree be modified by resistance changes in the peripheral vascular bed of the ischemic muscle region.

In contrast to its effect on CCBF, arterial blood pressure did not influence significantly the relative size of the ischemic region as shown by the comparison made in Fig. 6. In this figure the size estimates derived from the above series

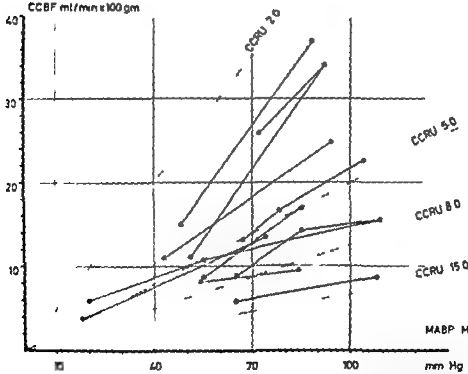

Fig. 4. Differentiated pressure flow relationships for the collateral vessels during occlusions of nine arteries. MABP – MRBP = mean arterial blood pressure minus retrograde pressure. Calculated collateral flow resistances (CCRU) ranged from 0.8 to 12.5 relative units.

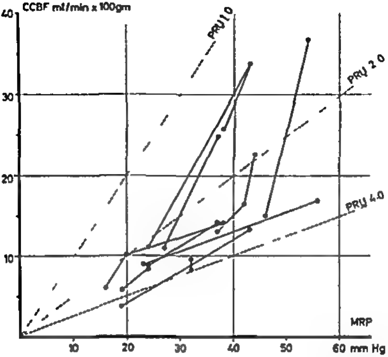

Fig 5 Pressure flow relationships for the peripheral vasculatures of the ischemic muscle regions resulting from the nine artery occlusions in Fig 4 MRP = mean retrograde pressure *Flow resistances of the peripheral beds (PRU) ranged from 1 4 to [illegible] relative units*

of arterial occlusions have been paired in a similar manner as in the heart rate series of Fig [illegible] The value obtained during occlusion of an artery at the highest mean blood pressure (abscissa) has been paired with one to four values obtained during occlusion of the same artery at lower pressure levels (ordinate) The sizes of the ischemic muscle regions in this material ranged from 19 to 86 per cent Fig 6 shows that the values for the relative size of the ischemic regions obtained during wide variations in blood pressure are distributed on both sides around the line of identity Mean differences between the values at the highest pressure and those at each lower level were not significant (M D -0.8 ± 1.4 S E, $r=0.94$) However in two experiments where blood pressure was lowered by *intravenous infusion of Arfonad* reduced *relative* sizes were calculated for the four ischemic regions (Fig 6) If they are excluded there is still no tendency to increased sizes of the ischemic regions at lower pressures (M D 1.2 ± 1.2 S E $r=0.93$)

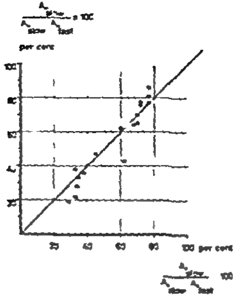

Fig. 6. Effect of arterial occlusion at different blood pressure on the resulting mass of ischemic muscle as estimated by the composite curve. The value obtained for an artery occlusion at highest mean pressure has been paired with one to four estimates at lower pressure levels resulting in 25 paired estimates out of the material in Fig. 3. ∘ signify the results in two experiments where Arfonad was infused intravenously. The mean difference was not significant even if the latter values are excluded ($r = 0.94$ and 0.93 respectively).

Discussion

For an analysis of the effects of heart rate and arterial blood pressure on myocardial circulation it is desireable to control a number of other cardio-vascular parameters. Of special interest with respect to collateral circulation are the possible changes in ventricular volume and pressure and their secondary effects on intramural pressure (HOLT 1957, LASZT and MÜLLER 1958, KIRK and HONIG 1964). For instance, the general response to an increasing heart rate produced by pacing of the heart is a decrease in stroke volume and a slight increase in aortic mean pressure at constant blood volume (RUSHMER 1956, BERGLUND *et al.* 1958). In the non failing heart these findings are conceivable with a reduced heart size at increasing frequency. This general response may be altered significantly when an acute coronary occlusion is added to the change in heart rate. In the present experiments there was sometimes an appreciable tendency to fall in blood pressure which had to be compensated for by transfusion from the pressure equilibrator with consequent alteration in blood volume and possibly also of heart volume and left ventricular diastolic pressure (HOLT 1957). The cardiac output determinations indi-

cated an unchanged or reduced stroke volume Due to the altered mechanics of the ischemic region an increase in residual volume is likely to occur This must be even more pronounced in case of a severe load on the ventricle by the actual occlusion In view of such considerations it shall be emphasized that the changes in CCBF reported in this study should be looked upon as the results of primary changes in heart rate and blood pressure together with secondary adjustments of other circulatory parameters

The results of variations in heart rate illustrated in Fig 1 above indicate a slight improvement of collateral blood flow when heart rate is reduced below 130 beats/min Comparable effects on retrograde flow were not observed by KATTUS and GREGG (1959) when heart rate was varied from 54 to 180 beats/min in two dogs They determined flow during 20 to 30 sec immediately after clamping the inflow catheter to the circumflex or anterior descending artery The collateral flow values reported in the present study are derived from the final part of desaturation curves recorded during arterial occlusions lasting at least 2 min This time difference could explain the divergency in the results In account of the small amounts of retrograde outflow within a fraction of a minute it may be difficult to detect small changes Actually when transformed into comparable figures the increase in CCBF reported here would be in the order of 1 ml/min or less This small increase may be compared with the value of 0 8 ml/min for the difference between duplicate determinations in the study by KATTUS and GREGG

The changes in CCBF reported in Fig 1 are likely to result from mechanisms other than true variations of tone in the collateral vessels It is well known that coronary blood flow under normal conditions to a large extent occurs in diastole since the vessels are markedly throttled by the high intramural pressures during systole (SABISTON and GREGG 1957 LEWIS COFFMAN and GREGG 1961) Such phasic variations can be expected also in the collateral blood flow into an ischemic portion of the myocardium which loses contractility and begins to bulge during systole within a minute after the onset of occlusion (TENNANT and WIGGERS 1935) The increase in CCBF at lower heart rates might therefore be explained by the prolonged diastolic period The data suggest that still lower heart rates might be optimal for the collateral circulation but the present study includes no results at heart rates below 90 beats/min Due to a progressive increase in diastolic volume more pronounced in the presence of ischemia it seems unlikely that CCBF would continue to go up at very low heart rates (see further below)

High arterial blood pressure is associated with a relative increase in CCBF in most of the present experiments (Fig 3) A close correlation between perfusion pressure and retrograde flow has been demonstrated by other

investigators (ANREP and HÄUSLER 1928 ECKSTEIN GREGG and PRITCHARD 1941 KATTUS and GREGG 1959) The general impression of the series of pressure flow relationships in Fig 3—5 is that the increase in CCBF is merely passive and that resistance to flow is little affected or slightly reduced due to distention On the other hand it was observed in a few cases that elevation of arterial blood pressure led to an increased resistance to collateral flow This occured in the most extreme form in the animal where CCBF was actually lower at a mean blood pressure of 197 mm Hg than at 66 mm Hg The markedly increased end diastolic pressure during the period of hypertension indicates a severe load and dilatation of the left ventricle The resulting elevation of intramural pressure over the whole heart cycle and elongation of the collateral vessels are likely to impede flow in the ischemic region This experiment is comparable to those of KATTUS and GREGG (1959) where they demonstrated the adverse effect of myocardial stretch on retrograde outflow

In general the present study indicates that raising arterial blood pressure improves collateral blood flow in the non failing heart

According to previous studies the coronaries of the mammalian heart represent a system of end arteries both from the physiological and the anatomical point of view (SCHLESINGER 1938 BLUMGART SCHLESINGER and DAVIS 1940 GREGG 1950 ZOLL WESSLER and SCHLESINGER 1951 BELLMAN and FRANK 1958 LIEBOW 1963) Collateral connections which bridge the terminal arborizations of neighbouring arteries are of arteriolar or precapillary size (PRINZMETAL *et al* 1947 BLUMGART *et al* 1950 BELLMAN and FRANK 1958 LAURIE and WOODS 1958) Moreover these collaterals are probably the only significant pathways conducting blood to an ischemic region after acute coronary occlusion in the healthy animal (GREGG and FISHER 1963) In view of these facts it is not surprising that definite changes in the relative size of the ischemic myocardial regions were not observed during variations in heart rate or blood pressure in the present study

The present study was supported by grants from Swedish National Association against Heart and Chest Diseases The authors acknoledge with thanks the technical assistance of Miss Maj Britt Johansson and the cooperation of Esbjörn Carlström Ph D in the statistical evaluation

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1966

THE SIGNIFICANCE OF THE CENTRAL CIRCULATION FOR THE AEROBIC WORK CAPACITY UNDER VARIOUS CONDITIONS IN YOUNG HEALTHY PERSONS

by

JESPER STENBERG

STOCKHOLM 1966

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The present thesis is based on data presented in the following papers

I Intra arterial blood pressure during exercise with different muscle groups
Pe Olof Astrand Bjorn Ekblom Roger Messin Bengt Saltin and Jesper Stenberg J Appl Physiol 1965 20 253—256

II Hemodynamic response to work at simulated altitude 4000 m
Jesper Stenberg Björn Ekblom and Roger Messin J Appl Physiol 1966 21 In press

III Hemodynamic response to work with different muscle groups sitting and supine
Jesper Stenberg Per Olof Astrand Björn Ekblom Joseph Royce and Bengt Saltin J Appl Physiol 1966 21 In press

The papers will be referred to by Roman figures I II and III

CONTENTS

PREFACE

In the course of many discussions with assistant professor Edvardas Varnauskas I developed during my student years a desire for scientific work.

I was introduced to the field of exercise physiology by assistant professor Per Olof Åstrand. The present investigations have been worked out under his guidance and partly in cooperation with him. It is a pleasure to acknowledge that my contact with him has been extremely rewarding from both a personal and scientific point of view. In my preparation of the papers and the summary professor Erik Hohwü Christensen generously proffered and indeed provided help with the formulation of my theme. For this I express my gratitude.

Several discussions with my collegues Bengt Saltin, Roger Messin, Björn Ekblom and Lars Hermansen have been of importance in the maturing of my thoughts in this field. — My gratitude is also extended to assistant professor Wilhelm von Döbeln who gave valuable advice concerning statistics. I want to express my thanks and admiration for patient and skilful assistance in the laboratory by Inger Hallbäck, Ulla Hollman, Brit Mari Fjellner-Ramning, Gunilla Sparell, Berit Pettersson Sjöberg and Gunnel Nordberg. I am also grateful to Mrs Karin Marina for help at the typewriter and to Mr Harry Hagelin for help in the workshop.

Without the tender help of my wife it is doubtful whether I had been capable of summoning the strength to complete my task.

The studies were supported by research grant from The Swedish National Association against Heart and Chest Diseases and The Swedish Sport Federation.

Stockholm 1966

Jesper Stenberg

INTRODUCTION

The aerobic work capacity can be defined as the highest oxygen uptake a healthy person can attain during physical work breathing air at sea level. A full utilization of the aerobic work capacity requires the engagement of large muscle groups such as in cycling or running. — The maximal oxygen uptake is a plateau value which can not be exceeded by increasing the work load. In cycling or running the maximal oxygen uptake is identical with the aerobic work capacity.

In the individual case aerobic work capacity shows slight variations from day to day (Åstrand et al. 1961 a). Interindividual differences are considerable, which can be explained by differences in maximal cardiac output and in the oxygen content of the arterial blood. It has thus been possible to show (Åstrand et al. 1964) that the correlation between the maximal oxygen uptake (measured on a bicycle ergometer) and the arterially transported oxygen (cardiac output times arterial oxygen content, both measured at maximal work load) was very high in a group of young healthy persons.

During arm work the maximal oxygen uptake is lower than that reached during leg work. In one case a lower cardiac output has also been demonstrated in maximal arm work than in maximal leg work (Christensen 1931 b).

In hypoxia the maximal oxygen uptake is lower than in normoxia. Part of the explanation is found in the lower oxygen content of the blood. Whether a lowering of the maximal cardiac output also occurs at acute exposure to hypoxia is not known.

In order to elucidate the importance of the central circulation for the maximal oxygen uptake in various conditions the following measurements were made:

I. Oxygen uptake, pulmonary ventilation, heart rate, cardiac output (dye dilution), intra-arterial blood pressure and arterial hemoglobin oxygen saturation and concentration have been measured in six healthy subjects who performed submaximal and maximal work on a bicycle ergometer in an altitude chamber at atmospheric pressure about 760 mm Hg and after acute lowering of the pressure to 462 mm Hg (corresponding to an altitude of 4000 m or 13000 feet).

II. The same functions were studied at sea level in sitting and supine position under submaximal and maximal work with legs, with arms and with arms and legs simultaneously in ten healthy, well trained subjects. — A complementary comparison between intra-arterial blood pressure during arm work and leg work in the sitting position was made in six untrained subjects.

istensen (1931 b) showed that the cardiac output is on the whole a rectilinear on of the oxygen uptake du ing work His results have later been confirmed eral authors as far as submaximal work is concerned However there is no atic study on the covariation between maximal oxygen uptake and maximal output until the work of Astrand et al (1964) In a group of twentythree young men and women with maximal oxygen uptakes between 2 4 and 5 1 and maximal ca diac outputs between 16 and 30 l/min the correlation n maximal oxygen up ake and maximal cardiac output was high but the tion was still higher between maximal oxygen uptake and transported oxygen maximal work where thus the arterial oxygen content is also taken into t

y few studies d al with maximal oxygen uptake and the circulation in dividual working under various conditions — Christensen (1931 b) showed subject that the maximal oxygen uptake in arm work was 3 0 l/min compared 7 l min in the same subject working with legs The cardiac output (acetylen d) was on these levels of oxygen uptake 25 3 l/min (heart rate 161 stroke 157) in the arm work and 35 1 l min (h art rate 169 stroke volume 209) in g work A lower m chanical efficiency in arm work was earlier describ d by the utho (1931 a) — Asmussen and Hemmingsen (1958) confirmed Christensens gs according to a low r mechanical efficiency and lower maximal oxygen uptake 1 work Individual data are not given but the authors claim that the maximal rate was the same in the eleven subjects studied whether p rforming arm work work — Ast and and Saltin (1961 b) found in three subjects that th maximal uptake in arm wo k averaged 70 % of that in leg work maximal h art rate ot reached in arm work In leg work supine position the maximal oxygen was 86 % of that reached in the sitting position In the combined work nd legs non of th five comparable subjects reached higher oxygen uptakes n wo k with only legs in sp te of larger muscle groups beeing engaged in the work

and (1952) showed in a g oup of 67 male and female subjects that there go d agre m nt betw en maximal oxygen uptake measured on a bicycle ergo and in running on a treadmill slope 1 75 % In the study of Astrand and (1961 b) three of th five subjects reached higher values on a treadmill (the ure suggest d by Taylor et al (1955) was followed) than on a bicycle re The differ nce averaged 5 % and was probably significant — One case is eported in which work with larger muscle groups elicited a considerably oxygen uptake than work with legs Christensen and Hogb rg (1950) and

Åstrand (1952 p 119) measured in skiing 5 2 l/min in a subject that could not exceed 4 5 l/min in cycling or running Liljestrand and Stenström (1920) found a similar but smaller difference in one subject when skiing and running were compared Taylor et al (1955) had a subject perform arm work during running on the treadmill with a speed and slope eliciting maximal oxygen uptake The added arm work gave a small but significant increase in oxygen uptake

In the literature there is a relatively large number of reports on the hemodynamic response to work at altitude in different stages of acclimatisation — The effect of acute hypoxia is studied mostly in resting subjects (cf Korner 1959) — There is no information available on maximal oxygen uptake in acute hypoxia — Christensen and Nielsen (1936) showed in one subject working in an altitude chamber (P_B 462 mm Hg a pressure corresponding to 4000 m or 13000 feet of altitude) that the cardiac output (acetylen method) was 29 % above the control value on a work load corresponding to an oxygen uptake of 1 l/min — Asmussen and Nielsen (1955) investigated the circulation of subjects breathing 12 % oxygen in nitrogen during graded exercise on a bicycle ergometer The hemoglobin oxygen saturation in arterial blood was between 65 and 70 % and the cardiac output (dye dilution) averaged between 10 and 20 % higher than the controls Unfortunately there are no individual data from this rather extensive study

EXERCISE IN ACUTE HYPOXIA

Material

Three of the six subjects were well trained athletes and three were sedentaries They were all familiar with the methods from participation in earlier studies

Methods

Exercise was performed on a bicycle ergometer in an altitude chamber described earlier by Åstrand (1954) Before the hypoxia experiments the pressure in the chamber was lowered to 462 mm Hg (corresponding to an altitude of 4000 m or 13000 feet) and it was kept constant The temperature in the chamber was between 18 and 22 centigrades the circulation of air was good and the composition of the air did not change during the experiments — When determining the cardiac output with dye dilution technique and a direct recording densitometer the method allows estimations of dye concentration independent of the degree of hemoglobin oxygen saturation provided the densitometer works at a wave length of 800 milli

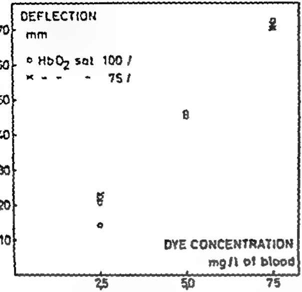


Fig 1 An example of calibration of the densitometer at two different levels of hemoglobin oxygen saturation in the blood

microns equally absorbed by reduced and oxygenated hemoglobin and equal to the peak spectral absorption of indocyaningreen (Cardio Green) Several measurements were done in vitro showing that the densitometer used measured concentrations of indocyaningreen in blood independent of the degree of oxygen saturation of hemoglobin see Fig 1

Procedure

The studies were done on two occasions two weeks apart On the first day 600 and 900 kpm min were done at simulated altitude or at sea level and thereafter the pressure was changed and submaximal and maximal work were done On the second day the order between hypoxia and normoxia experiments was reversed — The measurements began after 20 minutes in hypoxia and they were completed within 75 minutes in all cases

Results

Mean values are given in Fig 2 *Oxygen uptake* at submaximal work load was identical in hypoxia and normoxia Maximal oxygen uptake was however reduced in hypoxia to an average of 72 % of the control values *Pulmonary ventilation* (BTPS) was on the submaximal loads higher in hypoxia however the lower oxygen pressure in the inspired air was not fully compensated for which is revealed when

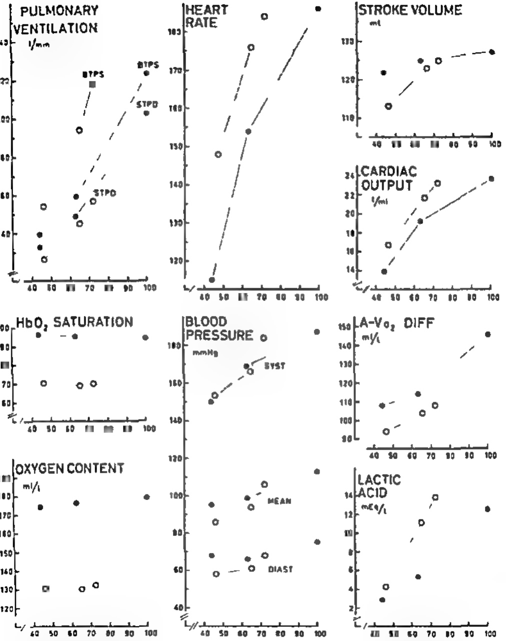


Fig 2 Circulatory and respiratory reactions to work in different work loads at sea level (closed symbols) and at simulated 4000 m (open symbols) as mean values from six young male subjects The scale on the abscissa refers to percentage of maximal oxygen intake on sea level

th pulmonary ventilation (STPD) is considered See the figure — In maximal work th pulmonary ventilation (BTPS) was nearly identical in the two different conditions — *The oxygen saturation* of the hemoglobin in arterial blood averaged 70 % in hypoxia The scattering was relatively large 62 to 82 % No tendency to decrease in saturations with increasing work load was traced — *The cardiac output* was in submaximal work loads significantly higher in hypoxia than in the controls At maximal oxygen uptake cardiac output was practically the same at hypoxia and normoxia The same was valid for the heart rate — The higher cardiac output on th lowest submaximal load was brought about by a higher heart rate and a lower *stroke volume* in the hypoxia experiments Th stroke volume had increased to control level at the high r submaximal load and therefore the heart rate determined the cardiac output

— The *arteriovenous oxygen difference* was lower in hypoxia the discrepancy be ng the largest in maximal work *Blood pressure* generally increased with increasing work load In average the mean blood pressures were lower in hypoxia than in normoxia The *total peripheral resistance* was in average lower at the lowest load in hypoxia than in normoxia In the higher work loads the tendency was the same but differences were not consistent and between mean values not significant

Discussion

In submaximal work the lower oxygen content in the arterial blood is not fully compensated for by the increased cardiac output in hypoxia the calculated mixed venous oxygen content was lowe on submaximal loads However the oxygen satura tion curve of hemoglobin is influenced by the lower pH suggested by the higher concentration of lactates in arterial blood in hypoxia so that the difference in oxygen tens on is small in the mixed venous blood which might be the goal for the regula tion of circulation

In maximal work the oxygen transpo t is already stressed to its limits — The pulmonary ventilation (BTPS) in hypoxia does not exceed that reached in no moxia and predicted values of maximal voluntary ventilation were not approached in the group studied Possibly the pulmonary ventilation in our subjects was regulated so as to reach an op imum in th balance between the benefit which a higher pulmonary ventila ion might have on the oxygen content in the arterial blood and the drawback of a high work of breathing for the total mechanical efficiency — The values of arterial oxygen saturations do not indicate falling diffusion in the alveoli neither hypoventilation with increased work load — Cardiac output reached the same highest level in hypoxia and normoxia with agreement in heart rates In chronic hypoxia a reduced maximal heart ate is found (Christensen and Forbes

1937 Astrand and Astrand 1958 Pugh 1964) which however increases when oxygen is administered (Astrand and Astrand 1958 Pugh 1964) — The low oxygen pressure and oxygen content in the arterial blood do not seem to influence cardiac performance With the lower mean pressure in the brachial artery which agrees with later findings (Blomqvist and Stenberg 1965) the work of the left ventricle is probably lower in hypoxia than in normoxia On the contrary the strain of the right ventricle will be highe supposing a higher pressure in the pulmonary artery during maximal work in acute hypoxia like in the resting state (Motley et al 1947) — In a comparable study including six very well trained athletes Blomqvist and Stenberg (1965) showed that there were no ECG signs of myo cardial ischemia when the subjects were performing maximal work at simulated altitude of 4000 m

The lower maximal oxygen uptake in moderate acute hypoxia compared with normoxia thus seems to be caused by the reduction of the arterial oxygen content During maximal work at hypoxia the oxygen uptake was 72 % the arterial oxygen content 74 % and the cardiac output 100 % of the control values in normoxia

EXERCISE WITH DIFFERENT MUSCLE GROUPS IN SITTING AND SUPINE

Material

In five well trained male subjects and one untrained determinations were made at submaximal and maximal work with the arms with the legs and combined arm and leg work in supine and sitting position In a female group including four well trained subjects the program carried out was similar but less complete The investigation was finally extended to a group of eight untrained subjects in which only the arterial blood pressure and the respiratory functions including oxygen uptake were measured in arm work and leg work in sitting position

Results

At the lowest work load the *oxygen uptake* was somewhat higher (lower mechanical efficiency) in arm work and combined work than in leg work With increasing load the oxygen uptake was the same in leg work and combined work at a given work load and lower than that in arm work The difference in this respect was most pronounced in the sitting position In sitting position maximal oxygen uptake in arm work reached 66 % of that in leg work for the males and for the females

71 %. The difference between maximal oxygen uptake in leg work and combined work was insignificant (see Fig. 3). The *pulmonary ventilation* attained in maximal arm work lower values than in leg work or combined work (see Fig. 4). Per litre oxygen uptake the pulmonary ventilation was however higher in arm work than in the two other types of work (see Fig. 7). Concerning the *cardiac output* there was no consistent

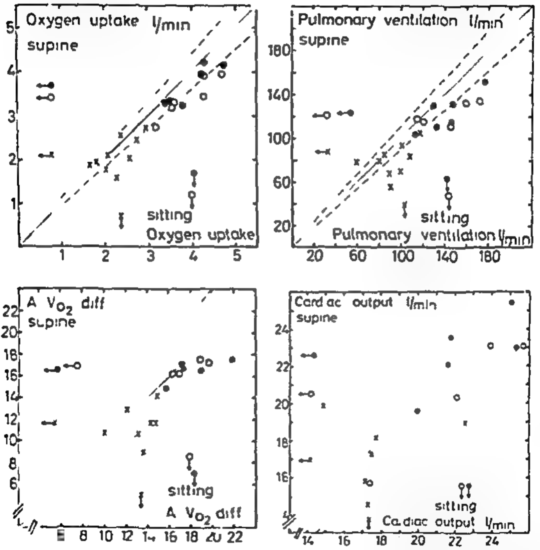


Fig. 3, 4, 5, 6. Comparison between highest values reached in sitting and supine position in arm work (x), leg work (o) and combined work (●). Line of identity and lines corresponding to ± 10 % deviation are drawn. Symbols with arrows give the mean of the different groups. — For the female subjects the above presentation was possible only for arm work, where mean values thus includes males and females.

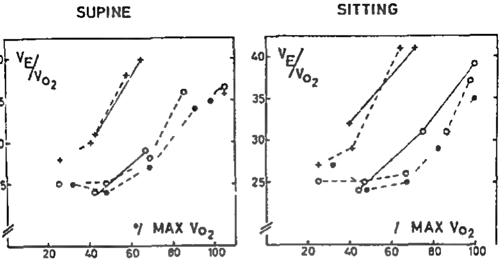


Fig 7 Pulmonary ventilation per liter oxygen uptake in relation to relative work load in arm work (x) leg work (0) and combined work (•) in male ( ) and female (——) subjects

difference between values from the three types of work in the same body position under submaximal work loads However the highest cardiac output attained in arm work sitting position averaged for males and females 80 % of that reached in leg work in leg work and combined work practically the same maximal values were attained see Fig 6 With the higher heart rates in arm work the calculated *stroke volumes* were smaller in this type of exercise 18 % lower when maximal values from arm work were compared with corresponding values from leg work, see Fig 8 — In supine position the combined work elicited the highest maximal cardiac output comparable with those in sitting position In leg work highest cardiac output averaged slightly lower than in supine and the stroke volume tended to decrease on work loads exceeding 60 % of highest oxygen uptake reached in any type of work here performed — A comparison between arm work and combined work performed in this body position shows that highest cardiac output can not be elicited by work with a relatively small muscle group — The highest values reached in arm work averaged 85 % of that attained in combined work the maximal oxygen uptake was only 65 % The stroke volumes were also significantly lower during arm work compared with combined work in supine position see Fig 8 — In arm work and leg work the *arteriovenous oxygen difference* was lower in supine position than in sitting but the difference decreased with increasing work load At maximal work the same high values as in leg work or combined work were not attained see Fig 5

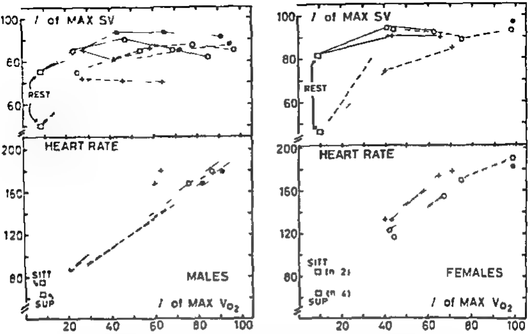
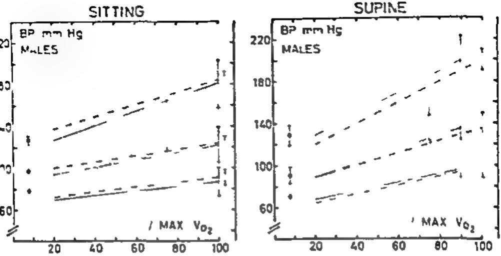


Fig. 8. Stroke volume (in % of highest stroke volume) and heart rate in relation to relative work load at rest in armwork (x), leg work (0) and combined work (●) sitting ( ) and supine (–). For female subjects the closed symbol corresponds to combined work, supine position. Values are given as mean of group values except for heart rate in the male subjects where regression lines are drawn.

The *blood pressure* increased with increasing work load in all types of exercise. Comparing submaximal loads the pressure in arm work was higher than in the other types of exercise; at maximal work the the highest values were reached in arm work in spite of a lower cardiac output (see Fig. 9). The total *peripheral resistance* was higher in arm work (see Fig. 10). The significance of the position of the intra-arterial catheter was pointed out in paper I. Slightly higher pressures were recorded from the resting limb than from the working.

Discussion

In this investigation it was necessary to study each subject on several occasions. This means that the results to some extent could be influenced by biological and methodological variations from time to time. Furthermore a relatively large number of work loads were performed at each occasion. It is known that heavy work influences the circulatory response to a subsequent lighter work (cf. Carlsten and



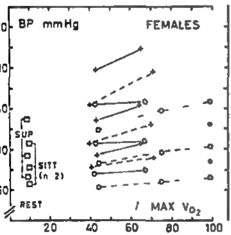


Fig. 9 Regression lines of systolic, diastolic and mean blood pressure on relative oxygen uptake in arm work ( ) leg work (——) and combined work ( ) in male subjects. Vertical lines symbol one SD. In female subjects mean values. Arm work (+) leg work (0) and combined work (●) sitting position ( ) and supine position (——)

Grimby 1966). In order to minimize the influence of the factors mentioned, the exercise was performed with increasing loads and the different types of exercise were alternated and on each experimental day exercise was done in sitting and in supine position.

The results confirm data in earlier reports concerning comparison between the types of exercise studied. Determinations of the cardiac output in supine and in sitting position have been reported by Bevegård et al. (1963) using the direct Fick method. The cardiac output averaged 2 l/min higher in the supine position than in sitting. The tendency was the same in the present material but less pronounced. The absolut values were lower here in both positions than in the study of Bevegård

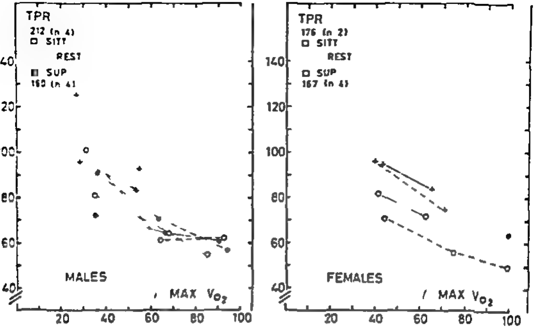


Fig. 10. Total peripheral resistance (arbitrary units) at rest and during arm work (+), leg work (○) and combined work (●) in sitting (- - -) and supine (——) position. — For female subjects the closed symbol corresponds to combined work in supine position. Values are given as mean of group values.

et al. (1963). Musshoff et al. (1959) investigated athletes in the supine position and the values of cardiac output (direct Fick method) agree well with the present results. The agreement with the results of Grimby and Nilsson (1963) (dye dilution method) is also good. — In sitting position the leg work and the combined work gave practically the same circulatory and respiratory response in submaximal and in maximal work. In the supine position the same high oxygen uptake was not attained in leg work as in combined work. A tendency to lower cardiac output, stroke volume and arteriovenous oxygen difference was observed in maximal legwork in supine position. The material is, however, to small for any definite conclusions to be drawn in this respect. In the studies of Musshoff et al. (1959) and Grimby and Nilsson (1963) a tendency to decrease in stroke volume at the highest work loads can be observed but not in Bevegård et al. (1963). It is possible that the relative load on the subjects is lower in the latter study.

Arm work differs under submaximal work from the other types of exercise in respect of mechanical efficiency, circulation and respiration. The cardiac output at a given oxygen uptake is the same as in leg work and combined work but the heart

rate is higher and thus stroke volume is lower. The higher heart rate was earlier pointed out and tentatively attributed to a less favourable distribution of the blood in arm work as a consequence of the fact that the large venous pump in the legs was not in action. It has now been shown that the difference in heart rate is still observable in lying position though not to the same extent. This could be interpreted in the following way. The more favourable redistribution of the blood simply caused by the supine position is not sufficient to keep up the large stroke volumes during work; there is also need for a large venous pump. In this material there is a significant difference between stroke volume in arm work and combined work in the supine position.

The difference between leg and arm work in supine position at highest work loads was, however, smaller and not significant. It is not certain that the conditions for venous return from the legs were inferior in arm work in this material. During heavy work with the arms a considerable supporting work in the legs was noticed. No attempt was made to evaluate objectively the role of the supporting leg work. Observations of the subjects and the personal experience of the authors suggest that there were rythmic isometric contractions of the leg muscles especially during the heavier arm works. Wang et al. (1960) could demonstrate that in the standing position stroke volumes could be brought up to the same levels as in supine position by isometric contractions of the calf muscles. Further Bevegård and Shepherd (1966) demonstrated that in leg work in supine position the resistance as well as the capacitance vessels were contracted in relation to the work load. Supposing that these findings are valid for the vessels in the legs during arm work in sitting position, then there are several possibilities to create a good venous return from the legs during arm work in our subjects.

The higher heart rate in arm work might be the primary reason for the low stroke volumes. The pulmonary ventilation is also higher in arm work and the mechanism behind the stimulation of these two functions might be the same. Irma Astrand et al. (1963) demonstrated in one subject that the cardiac output (acetylen method) was practically the same with a ventricular rate of 260 *beats per minute* in flutter as in normal rythm, heart rate 154. Similar results were demonstrated by Bevegård (1962) in patients with artificial pacemakers. — Further the higher blood pressure in arm work ought to be considered in this connection. Possibly the heart is regulated so that a certain cardiac output is brought about with a lower stroke volume in face of a higher blood pressure in the aorta, the stroke work thus beeing kept down. The investigation was not planned to elucidate the regulative mechanism behind the functions studied and therefore it seems essential to the author to point out that the explanations above are tentative.

The high blood pressure in arm work could be conditioned by a smaller vascular

to volume or mass The expected value of b was not found when heart volume was related to maximal oxygen uptake This suggest that some biological mechanism (s) is (a e) involved causing a more efficient oxygen transport in our subjects with a large heart volume — The author has now extended these calculations to the correlation between on one hand maximal oxygen uptake and on the other maximal cardiac output and maximal transported oxygen The b value 1 28 for the equation relating maximal oxygen uptake to cardiac output is different from the expected value 1 00 ($0\,1 > p > 0\,05$) Maximal oxygen uptake in relation to maximal transported oxygen gives a b value of 1 09 which is only slightly different from the expected 1 00 ($0\,3 > p > 0\,2$) The correlation coefficients were 0 88 (maximal oxygen uptake — cardiac output) and 0 95 (maximal oxygen uptake — transported oxygen) respectively — This approach to the problem of the determinants of the maximal oxygen uptake demonstrates numerically the significance of the maximal cardiac output and of the hemoglobin concentration (oxygen content) of arterial blood — The coefficient of oxygen utilization was slightly higher in the subjects with the higher cardiac outputs the male subjects 86 % compared with 83 % in the females and this might explain the slight deviation of the b value from the expected value when the maximal oxygen uptake was related to the transported oxygen in maximal work.

The experiments in hypoxia here presented elucidate further the relative importance of cardiac output and oxygen content of the arterial blood as determinants of the maximal oxygen uptake In spite of a low calculated Pa_{O_2} 40 mm Hg the cardiac output reached the same high values as in normoxia The maximal oxygen uptake was thus reduced to the same extent as the oxygen content of the arterial blood When maximal oxygen uptake was plotted against arterially transported oxygen in hypoxia and normoxia the points fall close to the regression line calculated from the study quoted above (Astrand et al 1964) where subjects with the higher capacity for oxygen transport were males and those with the lower capacity were females see Fig 11

Investigations carried out by Astrand and Astrand (1958) and by Pugh (1961) showed that neither the heart rate nor the cardiac output could reach the same high values at high altitude after acclimatization as on sea level before being exposed to altitude Sproule Mitch ll and Miller (1960) showed in anemic patients values of cardiac output of the same magnitude as those attained in a normal group performing maximal work on a treadmill Comparable studies concerning cardiac output and maximal oxygen uptake before and after acutely induced normovolemic anemia have not yet been reported

In order to elucidate the circulatory background to the reduced maximal oxygen uptake in exercise with small muscle groups arm work was chosen as a suitable

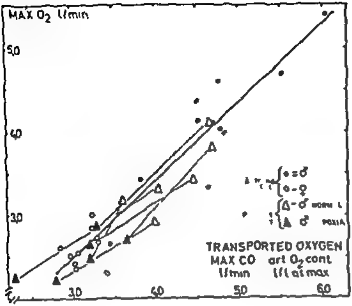


Fig 11 Maximal oxygen uptake in relation to the volume of oxygen transported per minute from the left heart during maximal work Open and closed circles and regression lines refers to Astrand et al (1964)

object for studies As earlier shown by Christensen (1931 b) in one single case the highest cardiac output attained in arm work was lower than that in highest leg work The highest attainable arteriovenous oxygen difference was also lower in arm work — The reason for the lower attainable cardiac output could be that exercise with a relatively small muscle group only stimulates the heart to a smaller extent In the sitting position however the combined work could not elicit a higher cardiac output than the leg work and in spite of the fact that the total load on the muscles was higher in the combined work the maximal oxygen uptake was practically identical — The higher blood pressure in arm work could possibly by some reflex prevent the cardiac output from increasing further and thereby increase the blood pressure — It is also possible that the cardiac work at maximal work had reached an optimal level in spite of the fact that the volume capacity was not reached

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STUDIES ON THE RELATIONSHIPS BETWEEN SOME METABOLIC EFFECTS OF TYROID HORMONES AND CATECHOLAMINES IN ANIMALS AND MAN

BY

NILS SVEDMYR

GÖTEBORG 1966

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This paper mainly constitutes a summary of the following articles

I SVEDMYR N The influence of thyroxine treatment and thyroidectomy on the calorigenic and some other metabolic effects of adrenaline and noradrenaline in experiments on fasted rabbits *Acta physiol scand* 1966 66 257–268

II SVEDMYR N Lactate elimination and oxidation in thyroidectomized untreated and thyroxine treated rabbits *Acta physiol scand* 1966 66 67–71

III SVEDMYR N Studies on the mechanism for the calorigenic effect of adrenaline in man *Acta physiol scand* (in press)

IV SVEDMYR N Metabolic effects of infused sodium L(+) lactate in man before and after triiodothyronine treatment *Acta physiol scand* (in press)

V SVEDMYR N The influence of triiodothyronine medication on some effects of adrenaline and noradrenaline in experiments on man *Acta pharmacol et toxicol* (in press)

VI HAGGENDAL J and N SVEDMYR The effect of triiodothyronine treatment on the catecholamine content of the blood during infusion of adrenaline in man *Acta physiol scand* 1966 66 103–105

VII SVEDMYR N and B WALDECK The effect of thyroxine treatment on the metabolism of tritium labelled catecholamines in the rat *Acta pharmacol et toxicol* 1965 23 225–230

These articles are referred to in the text as studies I II etc

CONTENTS

The following abbreviations have been used A=adrenaline NA=noradrenaline CA=catecholamines T_3=triiodothyronine T_4=thyroxine nic ac = nicotinic acid ^{3}H CA=tritiumlabelled CA FFA=free fatty acids Cyclic 3 5 AMP=cyclic adenosine 3 5 monophosphate

CHAPTER I

Introduction

The relationships between thyroid hormones and the adrenosympathetic system have been studied with increasing intensity for more than 100 years (for reviews see Griffith (1951) Ellis 1956 Hoch 1962 Harrison 1964 Rosenberg and Bastomsky 1965) An interrelationship has been found between many of the physiological pharmacological and biochemical effects of thyroid hormones and catecholamines (CA) The extent and the mechanism of this interrelationship has been the subject of many investigations but the results have been divergent and have not led to any generally accepted explanation

The stimulating effect of adrenaline (A) on the oxygen consumption (calorigenic effect) has been shown by many investigators to be potentiated by thyroid hormones both in different animal species (Abderhalden and Gellhorn 1925 Barker Fazikas and Himwich 1936 De Visscher 1946 Brewster *et al* 1956 Swanson 1956 1957) and in man (Horstman 1954 Murray and Kelly 1959) The calorigenic effect of A has been found to be diminished in thyroidectomized animals (Abderhalden and Gellhorn 1925 De Visscher 1946 *Brewster et al* 1956 Swanson 1956) *and in hypothyroidism in man* (Horstman 1954)

Brewster *et al* (1956) found in experiments on the dog that the stimulation of the oxygen consumption by thyroxine (T_4) was inhibited by sympathetic blockade produced by epidural anaesthesia They therefore assumed that the calorigenic effect of the T_4 was dependent on the activity of the sympathetic nervous system Adrenergic α and β blocking agents have only a moderate if any degree of influence on the calorigenic effect of T_4 however (Surtshin 1953 Holtkamp and Heming 1953 Ramey *et al* 1955 Schwartz 1962 Wilson *et al* 1964) and therefore the role of the sympathetic system in this connection has been doubted However it has been shown that guanethidine which inhibits the liberation of NA at the sympathetic nerve endings has some attenuating effect on thyrotoxic symptoms and reduces the oxygen consumption (Gaffney *et al* 1961 Lee *et al* 1962 Leak 1963 Waldstein *et al* 1964 Barker and Makiuchi 1965)

The calorigenic effect of A in the *rabbit* has been shown to be intimately associated with an increase in the production and metabolism of lactic acid

(Lundholm 1949 Lundholm and Svedmyr 1963 1964 Lundholm Mohme Lundholm and Svedmyr 1966) Brewster *et al* (1956) found that T_4 medication in the dog potentiated the stimulating effect of A on the lactic acid concentration in the blood while thyroidectomy diminished it Steinberg *et al* (1964) and Havel *et al* (1964) demonstrated that increased mobilization and metabolism of free fatty acids (FFA) were of importance for the calorigenic effect of NA in man Debons and Schwartz (1961) Deykin and Vaughan (1963) and Felt *et al* (1962 1963) showed that the FFA mobilizing effect of A was diminished in adipose tissue *in vitro* from hypothyroid but potentiated in adipose tissue from hyperthyroid rats Harlan *et al* (1962) found in man that that the FFA mobilizing effects of both A and NA were potentiated in hyperthyroidism and diminished in hypothyroidism but Hamburger *et al* (1963) were unable to verify this finding

It has not been established however if potentiation of the hyperlactacidemic and or FFA mobilizing effects of A contribute to the increase of A s calorigenic effect after administration of thyroid hormones

CHAPTER II

The present investigation

The aim of the present investigation was to study the influence of thyroid hormone medication on the calorigenic and some other metabolic effects of A and NA and the mechanism of this action. In the first studies (I and II) the way in which T_4 medication influenced the calorigenic, hyperlactacidemic, FFA mobilizing and hyperglycemic effects of infused CA was studied in the rabbit.

Since there was reason to believe that the mechanism for the calorigenic effect of A in man was not completely identical with that in the rabbit, studies were then made of the role of lactic acid metabolism and FFA mobilization in the calorigenic effect of A in man (studies III and IV) and also of the influence of thyroid hormone administration on the metabolic effects of A and NA (study V).

The above studies indicated that thyroid hormones increased the stimulatory effect of A on lactate production and metabolism and that this action was one of the more important factors for its potentiation of the calorigenic effect of A.

These findings gave rise to futher questions of the ways by which thyroid hormones induced potentiation of the hyperlactacidemic and calorigenic effects of A. There appeared to be two possibilities *a priori*. 1) Inactivation of infused A was inhibited by thyroid hormones whereby the A concentration at the receptors increased and/or its action was prolonged. 2) Thyroid hormones sensitized the adrenergic effector cell so that the same A concentration gave a stronger effect (a) by sensitization of the adrenergic receptor, (b) by stimulation of one or more normally rate limiting links in the biochemical reaction chain which was stimulated by A; the point of attack was assumed to be situated below the primary reaction between A and the receptor. A combination of these possibilities was also conceivable.

In studies VI and VII alternative 1 was investigated. No evidence was found to support this hypothesis, however, and it was assumed that the probable cause of the potentiation lay in alternative 2. In a series of studies (Svedmyr 1965 a, b, 1966 a, b) alternatives 2 a and 2 b are being investigated. The results hitherto obtained from these latter studies will be discussed briefly in this review.

CHAPTER III

Materials and methods

Rabbit experiments

For the metabolic studies on animals (studies I and II) adult male rabbits were used. They have been found to have a particularly constant basal O_2 consumption and are easily prepared in an unanesthetized state. They were quiet during the entire experiment and in addition their blood volume was sufficient for these studies.

A catheter was introduced into a marginal ear vein for infusion of CA and another catheter into a central ear artery for the withdrawal of blood samples. When the catethers were not used for these purposes physiological saline at a rate of 10 ml/hour was infused into both of them. The rabbits were then placed in a metabolism cage (Fig. 1) which was made for these experiments being a modification of an apparatus described by Lundholm (1949).

The oxygen consumption in control animals in which only physiological saline was infused remained unchanged as also did the blood lactate concentration. Thus the standard deviation for change in the oxygen consumption between 60 minute periods in the same animal was only $\pm 1.34\%$ in tests on 5 animals during a period of 4 hours (n=20).

The rectal temperature was separately studied in some experiments. At a cage temperature of 24° C some of the T_4 treated animals developed a hyperpyrexia. Therefore the cage temperature was kept at 18° C$\pm$0.1°. At this cage temperature the maximal increase in rectal temperature was 1° C and about the same in the different groups.

Rat experiments

For determination of the CA metabolism adult male rats were used to reduce the cost since ^{3}H CA preparations are very expensive. Moreover this is one of the most common animal species used for such investigations which simplifies comparison of the results. The size of the blood volume in this case was of no importance.

Experiments on man

Male subjects who had previously taken part in similar experiments and who had fasted since the evening before were used. The O_2 consumption

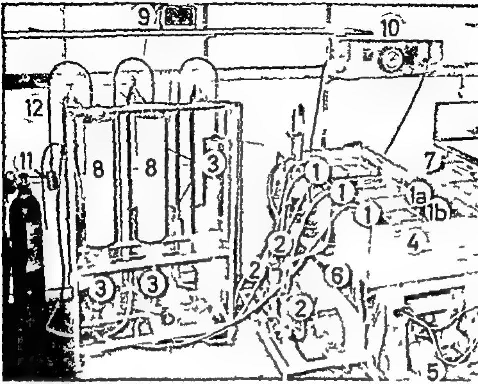

Fig 1 The author's *modification of an apparatus for determination of the oxygen consumption in small animals by the closed circuit system* (Benedict 1915) The metabolism of the rabbit and rat was determined with this apparatus

The apparatus comprises three separate metabolism cages (1) each with a circulation pump (2) and spirometer (3) Each cage consists of an animal section (a) and a section (b) containing superimposed on one another absorption media for aqueous vapour ("blue gel") and carbon dioxide (soda lime) The cages are immersed in a water bath (4) the temperature of which can be kept constant at 18 ± 0.1 C The water circulates through a circulation pump (5) and the water level which during the course of the experiment lies about 3 cm above the lid of the cages can be lowered by running the water out into a special container (6) This can be emptied by means of a three way tap via the circulation pump (5)

The animal can be observed during the entire experiment through the transparent plexiglass lid of the cage Catheters can be passed via cannulae in a rubber cork from an artery and a vein in the rabbit's ear for example to an infusion pump (7) blood samples can be taken from the artery and the substance to be studied can be infused into the vein For the remaining time the catheters can be kept patent by slow infusion of 0.9% NaCl solution

By the circulation pumps (2) the air is sucked from the animal section in the metabolism cage through the absorption media and then returned A rubber tube connects the metabolism cage to its respective spirometer (3) The spirometric reduction is recorded on a kymograph with electrosensitive paper (8) by means of a generator (9) By means of the

clock (10) the kymograph cylinder moves forward approximately 8 mm at intervals of exactly 10 minutes. The spirometers are balanced by a counter weight (11) and a plastic tube filled with mercury (12) with such dimensions that it balances the spirometer exactly in all positions. The entire apparatus stands in a room thermostatically controlled at 16 ± 0.5 C.

CO_2 production and ventilation were measured with a Hartmann and Braun's metabolic apparatus (Frankfurt/Main, Germany). The analyzer measures continuously the difference in the O_2 and CO_2 contents of inspired and expired air by variations in heat conduction capacity. The values were recorded with a Cr... instrument. The standard deviation of the oxygen consumption between two basal 10 minute periods in the same subject was ±2.47 per cent (n=25). The body temperature was measured in some tests and a maximal increase of 0.15 C was recorded after infusion of CA.

Chemical analyses

The blood lactate concentration was determined in a number of rabbit experiments according to Friedemann and Graeser (1933) and in the remaining experiments by an enzymatic method described by Lundholm, Mohme-Lundholm and Vamos (1963). The accuracy of these methods has been investigated in a special study (Lundholm, Mohme-Lundholm and Svedmyr 1963). The standard deviation for the method of Friedemann and Graeser was ±3.5% and for the enzymatic method ±3.1%. The blood glucose concentration was determined enzymatically according to Bergmeyer and Bernt (1962), the standard deviation was ±3%. The plasma FFA was determined according to Trout, Estes and Friedberg (1960) with a standard deviation of ±5%.

Materials

L(−)lactate (Fluka) in the form of sodium salt with a pH of 7.40 was used. On polarimetric analysis and determination of the water content in the zinc lactate, this was found to consist to at least 99% of the L(+) form. Sterile and pyrogen free lactate solution was prepared and tested by the pharmaceutical research department of AB Bofors.

The purity of the respective ³H−CA preparations (from New England Nuclear Corporation, Boston) was determined by paper chromatography. The T_4 and T_3 were prepared by AB Vitgaard. T_4 was dissolved in sterile pyrogen free 0.9% physiological saline the pH of which was maintained at 11 by sodium hydroxide.

Synthetic L-adrenaline (ACO) and L-noradrenaline (Astra) were used. The CA were diluted with physiological saline to which was added 0.1 per cent ascorbic acid.

Choice of thyroid hormone dose

The physiological and biochemical effects of thyroid hormones are in many cases dependent on the dose and on the duration of the medication (Hoch 1962) Opposite effects for example on the glycogen content in the liver can be obtained with low and very high T_4 doses (Wertheimen 1953 1956) Very high T_4 doses also act as uncoupler of the oxidative phosphorylation in contrast to more physiological doses (Tata 1965)

It is probable that high thyroid hormone doses which result in considerable loss of weight may also influence the activity of other endocrine organs so that the relation between CA and thyroid hormones may be complicated further In consideration of the risk of such secondary effects the thyroid hormones were given in doses low enough and for periods short enough for the body weight of the animal to be maintained and for excessive thyrotoxic symptoms to be avoided In the experiments in man the dose chosen was such that the subject was able to lie in a relaxed state during the whole experiment despite the pulse increase and nervousness that could be expected during A infusion

Fasted animals and human subjects

The calorigenic effects of A (v Euler and Liljestrand 1927) and NA (Havel *et al* 1964) have been stated to be diminished in non fasting human beings These findings suggested that the present experiments should be performed under fasting conditions Moreover this made it possible to relate changes in oxygen consumption to a basal unchanging state The observed relationships between CA and thyroid hormones in fasted individuals cannot be assumed to be exactly the same under nonfasting conditions

Statistical analyses

Student s t test as well as regression analyses as given by Bonnier and Tedin (1957) were used

CHAPTER IV

The influence of thyroxine treatment and thyroidectomy on the calorigenic and certain other metabolic effects of adrenaline and noradrenaline in fasting rabbits

Preliminary experiments in the rabbit had shown that A had a marked increasing effect both on the lactic acid and FFA concentrations in the blood The FFA increasing effect of A was of a transient nature however, and disappeared in spite of continuation of A infusion The effect of NA on the blood lactate concentration was considerably weaker than that of A Its effect on the plasma FFA concentration was initially of similar magnitude to that of A but was of longer duration By studying the way in which T_4 medication influenced the calorigenic effect of both A and NA a possibility was obtained of gaining some idea of the relative importance of lactic acid production and FFA mobilization for the potentiation of the calorigenic effect of CA by T_4

In experiments on rabbits which had been given daily subcutaneous injections of 1 mg L T_4 for 7 days the basal oxygen consumption increased by 45–65 per cent in different groups of animals Neither the basal lactate glucose or FFA concentrations were influenced by this treatment On infusion of 0 5 µg A/kg/min for 60 min the calorigenic effect expressed as increase of O_2 consumption over basal value (in ml /kg/min) was approximately twice as great after T_4 treatment as before (Fig 2 study I Table 1) The elevating effect of A on the lactate concentration in the blood was significantly greater after T_4 medication while its hyperglycemic effect on the other hand was diminished The FFA increasing effect in plasma remained unchanged

The calorigenic effect of NA given in a dose of 0 5 µg/l g/min for 60 min, was only about 35 per cent of that of A in untreated animals (Fig 2 study I Table 1) The effect of NA on the blood lactate concentration was a little below 1 10 while the hyperglycemic effect was approximately 1/6 of the A effect On the other hand the effect of NA on the plasma FFA concentration was equal to but of longer duration than the A effect T_4 treatment did not influence the calorigenic effect of NA or its stimulation of the blood lactate or glucose concentrations and neither did it alter its FFA stimulating effect significantly

These results indicated that only the calorigenic and hyperlactacidemic effects of A were potentiated by the T_4 treatment In a further study the

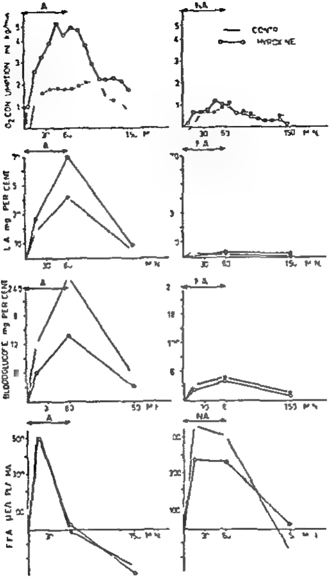

Fig 8 The influence of thyroxine treatment on the calorigenic hyperglycaemic hyperlactacidemic and lipolytic effects of adrenaline (A) and noradrenaline (NA) in experiments on fasted rabbits The catecholamines were given i v 0 5 μg/kg/min for 60 min Each point represents the mean increase over basal value of 8 expts.

influence of thyroidectomy and T_4 treatment on these effects was therefore investigated more closely Animals which had been thyroidectomized at least 4 weeks before the experiment had a basal metabolism which was approximately 20 per cent lower than that of normal controls The calorigenic and

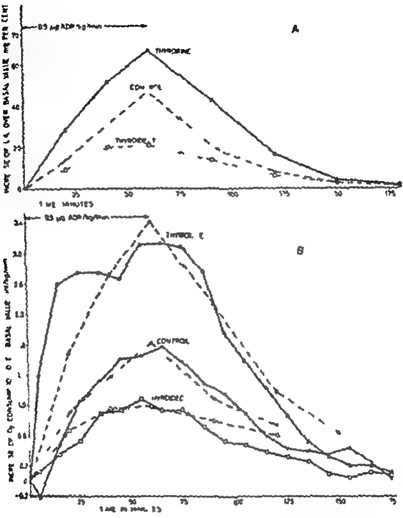

Fig. 3 A. The effect of adrenaline infusion (0.5 μg/kg min for 60 min) on the lactic acid (L.A.) content of the blood in untreated (control, n=8), thyroidectomized (n=10) and thyroxinetreated (n=8) rabbits. Mean increase over basal value.

B. The effect of adrenaline infusion on the oxygen consumption in the same experiments as in Fig. 3 A. Oxygen consumption in ml/kg min above basal values. The continuous curves show the values obtained in the experiments. The dotted curves represent the expected increase in oxygen consumption which should occur as a result of increased lactic acid metabolism. These expected increases in oxygen consumption have been calculated from the following regression equations found in Study II. Untreated rabbits Y = 0.0[illegible] X + 0.[illegible]. Thyroidectomized rabbits Y = 0.0[illegible]6 X − 0.44. Thyroxinetreated rabbits Y = 0.0[illegible] X [illegible] 0.43. Y = increase of oxygen consumption over basal value in ml/kg/min. X = increase of lactic acid content in the blood over basal value in mg per cent.

hyperlactacidemic effects of A were significantly lower in these animals than in normal animals (Fig 3 study I Table II)

The potentiated effect of A on the blood lactate concentration in T_4 treated animals could be due to increased lactic acid production in the tissues or to decreased elimination of lactic acid or possibly to a combination of these factors The diminished A effect after thyroidectomy could in turn be due to a decreased production and/or increased elimination It was necessary for

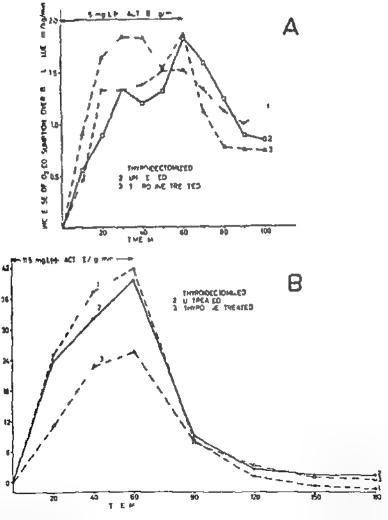

Fig 4 The effect of infusion of 11 5 mg L(+) lactate as sodiumsalt on the oxygen consumption and on the lactic acid content of the blood in untreated thyroidectomized and thyroxinetreated rabbits Mean increase over basal value of 5 experiments

the subsequent analysis to attempt to establish which of these alternatives was correct.

In order to quantitatively reproduce the lactate increase which occurred in the blood during the A infusion, L(+)lactate in the form of Na salt was infused intravenously in a dose of 11.5 mg/kg/min for 60 min in untreated, T_4 treated and thyroidectomized rabbits, and the effect on the blood lactate concentration and the oxygen consumption was determined (Fig. 4, study II, Table I and II). In untreated animals the blood lactate concentration increased to approximately the same extent as during the A infusion. After an infusion period of 60 min the lactate increase in the blood was 40 mg per cent during lactate infusion and 43 mg per cent during A infusion. In T_4 treated animals lactate infusion induced a significantly smaller increase of the blood lactate concentration than in untreated and thyroidectomized animals both of which showed similar values. Obviously lactate was eliminated more rapidly from the blood after T_4 medication. On the other hand the increase in oxygen consumption appeared to be initially somewhat greater in T_4 treated animals. It seems probable therefore that the increased elimination was associated with an increased lactate oxidation.

Since the elevating effect of A on the blood lactate concentration was potentiated by T_4 medication, this can only be interpreted as that T_4 increased the lactic acid producing effect of A in the tissues. On the other hand the diminution of the hyperlactacidemic effect of A which was induced by thyroidectomy must be ascribed to a decrease in the production of lactic acid in the tissues.

A regression analysis was performed of the values in the lactate infusion test in order to get a more quantitative evaluation of the relationship between the increase in blood lactate concentration and that in oxygen consumption over the 10 min period during which the blood sample for lactate determination was taken. A significant correlation was found for all three animal groups (study II, Table III). This relation tended to over-estimate somewhat the effect of a low lactic acid increase on the O_2 consumption, but probably underestimated the effect of higher lactate increase.

If it is assumed that the stimulation of the O_2 consumption associated with an increased blood lactate concentration *per se* was of the same magnitude on infusion of A as on infusion of lactate, the O_2 consumption stimulation resulting from the hyperlactacidemic effect of A can be calculated. By means of the regression equations in the text to Fig. 3B (or study II, Table III) the probable lactic acid induced increase in O_2 consumption during A infusion was calculated for the different groups from the observed increase in blood lactate (Fig. 3B, study I, Table II). These calculated values closely agreed

with the total O_2 consumption found experimentally during A infusion (Fig 3B) It is possible that the role of lactate metabolism is somewhat over estimated in this calculation

These results indicate however that the variation demonstrated in the calorigenic effect of A in untreated T_4 treated and thyroidectomized animals can be ascribed to a large part to variations in the rate of lactic acid production and the intensity of its metabolism

Both A and NA had an FFA mobilizing effect in the rabbit (study I Table I) In man this effect was associated with a calorigenic action (study III) It is probable therefore that this lipolytic effect may have played a certain part in the calorigenic effect of CA in rabbits also The FFA increasing effect of A in plasma was however considerably weaker in the rabbit than in man (Fig 2 study I Table I Fig 6 study III Table II) By selective blockade of the lipolytic effect of NA with nicotinic acid in the rabbit Svedmyr and Lundholm (1966) found that the calorigenic effect of NA was thereby reduced by about 30 per cent This indicates that the calorigenic effect of NA has some connection with its lipolytic effect while in the rabbit this latter effect is probably of minor importance for the calorigenic effect of A

T_4 medication however did not potentiate either the calorigenic or the lipolytic effects of NA This appears to indicate indirectly that the lipolytic effect was of no great importance for the potentiation of the calorigenic effect of A by the T_4 either The influence of thyroid hormones on the FFA mobilizing effects of CA is discussed further in connection with experiments in man in chapter V

CHAPTER V

Studies on the mechanism for the calorigenic effect of adrenaline in man

The mechanism for the calorigenic effect of A in man is as yet not clearly known It was therefore considered desirable to study this mechanism in more detail especially the relative importance of the lactic acid and FFA metabolism before the effect of thyroid hormones on the calorigenic effect of C was investigated The problem has two aspects firstly the metabolic background to the calorigenic effect of A and secondly the question of in which organ or organs the calorigenic effect takes place The investigations described below mainly concern the first aspect while the second aspect is treated more in the form of discussions with reference to previous studies Following the investigations of Lundholm (1949) on the importance of lactate metabolism for the calorigenic effect of A in the rabbit Bearn Billing and Sherlock (1951) demonstrated in man that A infusion more than doubled the oxygen consumption of the liver At the same time as the blood lactate concentration rose the uptake of lactate by the liver increased NA infusion increased the oxygen consumption of the liver approximately 1/3 as much as A infusion The FFA metabolism was not investigated These experiments appeared to show that an increase in lactate metabolism could play a part in the calorigenic effect of A in man also On the other hand NA had very little effect on the lactate concentration in man despite a definitely calorigenic effect (Cobbold and Ginsburg 1960 Lundholm and Svedmyr 1966)

The CA induced increase of the FFA concentration in plasma first demonstrated by Dole (1956) has been assumed however to be of importance for the calorigenic effect of NA and NA has been shown to increase the oxidation of infused C^{14} labelled palmitic acid (Steinberg 1963 Havel 1964) Steinberg *et al* (1964) showed that the adrenergic β blocking agent pronethalol completely blocked the FFA increase and the calorigenic effect after NA administration Adrenergic β receptor blocking agents also block the phosphorylase activating and hyperlactacidemic effects of CA and their stimulatory effects on the heart and respiration however (Lundholm and Svedmyr 1963 1966 Mohme Lundholm and Svedmyr 1964 Svedmyr 1966) and therefore no definite idea of the contribution of FFA mobilization alone to the calorigenic effect of CA can be obtained from experiments with β adrenergic blocking agents Havel *et al* (1964) selectively blocked the FFA mobilizing

effect of NA with nicotinic acid however whereby the calorigenic effect of NA was reduced to half Increases in both FFA and lactate metabolism could therefore be assumed to be factors of importance for the calorigenic effect of NA in man

The effect of A infusion In an attempt to determine the relative importance of these factors for the calorigenic effect of A a series of experiments were performed on human subjects where the effect of an infusion of A (0.10 µg/kg/min for 30 min) on the O_2 consumption was studied The CO_2 production and respiratory volume were also recorded At the same time the influence on the blood lactate and glucose concentrations and on the plasma FFA were studied and the systolic and diastolic blood pressure and the pulse rate were recorded

The influence of A on these parameters is shown in Fig 5 and 6 (and study III Table I and II) The O_2 consumption showed a considerable increase as early

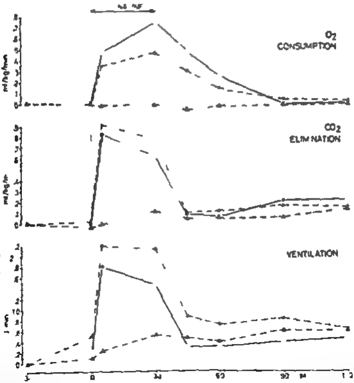

Fig 5 Influence in man of A (— — — —) nicotinic acid (Δ — — — — Δ) and nicotinic acid combined with A (o———————o) in i v infusion on O_2 consumption CO_2 elimination and ventilation A in a dose of 0 10 µg/kg/min between 0 and 30 min Nicotinic acid in a dose of 5 mg/min between −30 and 120 min Every point is the mean of 5 experiments

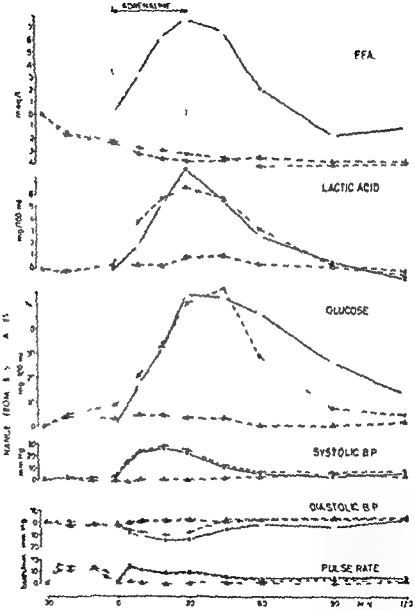

Fig. II. Influence of A (— — —) nicotine and (Δ— — — —Δ) and nicotine combined with A (o — — — —o) on some metabolic and circulatory functions in For further informations see Fig.

as after 5 minutes and then continued to rise reaching its maximal value after 30 min infusion corresponding to an increase in the basal O_2 consumption by about 30 per cent. The increased value was maintained even after the end of the A infusion and the basal value was not regained until 60 min after the infusion had ended. The CO_2 production already reached its maximal value after 5 min infusion when it exceeded the O_2 consumption. This meant an increase in the respiratory quotient (R. Q.). The reason for this has been studied and discussed by Lundholm and Svedmyr (1965, 1966). It is probable that about fifty per cent of the increase in CO_2 production was due to the fact that CO_2 was driven out of the blood and tissue bicarbonate as a result of the increase in organic acids (lactic acid, FFA, hexosephosphoric acids, etc.) induced by A in the blood and tissues. These factors must be taken into consideration when interpreting R. Q. changes during A infusion.

The ventilatory changes ran parallel with those of the CO_2 production.

A increased the lactate concentration in the blood but the maximal increase was fairly moderate (8 mg per cent in this study and 9 mg per cent in another similar series of experiments in study V). The FFA increase, on the other hand, was considerable (700—800 μE/l) and a moderate hyperglycemia (with an increase of 50 mg per cent) was also noted. The metabolite concentrations in the blood reached their maximum at the same time as the oxygen consumption.

The pulse rate rose (by 15 beats/minute) at the start of the A infusion. The systolic blood pressure increased and the diastolic decreased during and after the infusion (Fig. 6).

The role of FFA mobilization in the calorigenic effect of A

In further experiments the FFA mobilizing effect of A was blocked by nic. ac. As a control the effect of nicotinic acid alone, infused in a dose of 5 mg/min for 150 min, was studied. In order that the subjects should become accustomed to the nic. ac. "flush" and should acquire some tolerance to it, an oral dose of 0.5 g of nic. ac. was given three times daily for [illegible] days prior to the experiment. The effects of nic. ac. infusion on the different metabolic and circulatory functions are shown in Figs. 5 and 6 (and study III, Tables I and II). It is seen that the basal O_2 consumption remained unchanged during the nic. ac. infusion. This is in agreement with the results of Havel *et al.* (1964) but contradicts those of Dagianti and Chiavaro (1964) who states that nic. ac. increased the O_2 consumption. In the latter study nic. ac. was given in a single intravenous dose, however, and apparently without previous nic. ac. medication so that flushing and other subjective symptoms, e.g. paresthesias and pyrosis, were probably pronounced.

After 60 min infusion nic. ac. elevated the CO_2 production somewhat, so

however in establishing the relationship between the increase in O_2 consumption and the rise in lactate concentration in the blood

It was found in the present experiments that after 30 min infusion of 23 mg/kg/min L(−) lactate (as sodium salt) the lactate concentration in the blood increased by approximately the same amount as on infusion of A (Fig. 7 study IV Table II) This lactate value also agreed with that which can be calculated from the maximal lactate elimination from the forearm musculature which was found by Lundholm and Svedmyr (1965) to be induced by a corresponding A infusion The lactate infusion induced a maximal increase of 10 per cent in the O_2 consumption Another observed effect was a slight increase of the plasma FFA concentration (Fig 8)

Discussion

In order to make a more quantitative estimate of the relationship between the increase in the O_2 consumption (Y in ml/kg/min) and the increase in the lactate concentration in the blood (X in mg per cent) over the basal value a regression analysis of the lactate infusion experiments was performed The regression equation $Y = 0.028X + 0.032$ was obtained The correlation between X and Y was statistically significant (Table III study IV)

By means of this equation the increase in oxygen consumption that could be expected to be associated with the increase in blood lactate concentration during the A infusion was calculated in the same way as in the experiments on the rabbit This calculated value marked as *L A* in study III Fig 3 was 35 per cent of the total calorigenic effect of A

An interesting question is how and where an increased FFA and lactate metabolism stimulate the O_2 consumption Since available experimental data are partly insufficient and partly controversial the following discussion is somewhat hypothetical

FFA mobilization The evaluation of the role of FFA mobilization in the calorigenic effect of A is based on the fact that this mobilization and part of the calorigenic effect of A are blocked by nic ac It would be of value if the plasma FFA concentration could be increased to the same level as during the A infusion by infusion of fatty acids It has been estimated however (Dole 1961 Fritz 1962) that half the quantity of FFA in plasma leaves the blood every alternate minute and therefore considerable amounts of FFA would have to be administered Free fatty acids are quite toxic however (Oro and Wretlind 1961) and cannot therefore be given in large quantities as sodium salts Attempts have been made in the rabbit to give palmitic acid in a 1 per cent emulsion together with lecithin (Svedmyr unpublished) In

the small doses which were tolerated by the animal without toxic symptoms this infusion induced only a minor increase of the FFA concentration in the blood It has also been claimed (Fritz 1961) that FFA in such an emulsion is utilized by the tissues to a smaller extent than protein bound FFA One alternative would be to give proteinbound fatty acids but in that case large quantities of solution would have to be infused which would probably result in side effects other than those due to the FFA

It has been demonstrated that an increase in the FFA concentration in plasma or in the suspension solution in *in vitro* experiments may lead to increased FFA oxidation (Fritz 1961 Steinberg 1963) but in most experiments without influencing the total O_2 consumption Challoner and Steinberg (1966) have however recently found that an increased FFA concentration in the perfusate increased the O consumption of isolated rat heart

According to Dole (1964) only 10—15 per cent of the FFA that leaves the plasma will be oxidized on excessive FFA mobilization while the remainder may be assumed to be resynthesized to triglycerides *inter alia* According to Steinberg *et al* (1964) and Havel (1964) NA increased the turn over rate of FFA Carlson *et al* (1965) have demonstrated that the FFA mobilizing effect of NA was accompanied by an increase in the triglyceride content in *inter alia* the liver and muscle The resynthesis of FFA to triglycerides requires α glycerophosphate (Fritz 1961) which the cell derives via the glucose metabolism An increased glucose concentration in the blood reduces the plasma FFA concentration and increases the resynthesis of triglycerides (Fritz 1961 Steinberg 1963) The hyperglycemic and FFA mobilizing effect of A can therefore be assumed to be associated with a triglyceride resynthesis The calorigenic effect of A could therefore partly be due to stimulation of an FFA cycle with an increase of both hydrolysis and resynthesis of triglycerides This cycle is energy consuming and it has been suggested (Ball and Jungas 1961 Havel 1964 and Masoro 1966) to be partly responsible for the calorigenic effect of NA

Further studies of this problem are however required in order to determine more quantitatively the relative importance of direct FFA oxidation and/or the resynthesis of FFA to triglycerides for the calorigenic effect of CA

Lactate metabolism On infusion of L(+) lactate in man (study IV) the total O_2 consumption increased to an extent corresponding to total oxidation of one third of the infused lactate quantity

According to the classic studies of Cori (1931) A stimulates a lactate cycle whereby the glycogen in the muscle is broken down to lactate which is transported via the blood to the liver where a large part of it is resynthesized to glycogen or glucose and a part is oxidized This reaction is energy consuming

and it is probable that the oxygen consumption in the liver is increased by the oxidative and synthesizing reactions.

Bearn *et al.* (1951) found in experiments on man that during infusion of A the lactate uptake of the liver increased and that its O_2 consumption was doubled. As NA stimulated the oxygen consumption of the liver to about 1/3 of that of A, it is probable that other factors beside increased lactate metabolism were of some importance too. Thus an increased FFA uptake and metabolism may also have contributed.

Experiments by Drury and Wick (1965) have, however, given rise to some doubt about the extent to which lactate is resynthetized to glycogen in the liver. After injection of C^{14} lactate only 4—5 per cent of the radioactivity was found in the liver glycogen while about 65 per cent was oxidized to $C^{14}O_2$. The liver was the most important organ for oxidizing lactate but some lactate was also metabolized in other organs. Schimassek (1965) demonstrated that A increased the lactate uptake and oxygen consumption of the isolated liver. Exton and Park (1965) have found that cyclic 3—5 AMP and hormones stimulating formation of this nucleotide (glucagon and A) also stimulated the conversion of lactate to glucose in the liver. It is therefore possible that A may increase the part of lactate that is resynthesized to glycogen and glucose.

It is evident from Fig. 11 that an increase in the FFA and lactate metabolism can only partly explain the calorigenic effect of A, and that a remaining part is due to other factors. The magnitude of this 'residual effect' is open to discussion, however.

One factor of some importance for this residual effect appears to be the hyperglycemic effect of A. Boothby and Sandiford (1923) found that an experimental hyperglycaemia which, however, exceeded that induced by A, increased the O_2 consumption in man by about 5 per cent. It seems probable that the hyperglycaemic effect of A can be expected to give an increase of 2—3 per cent in the total O_2 consumption. This would correspond to about 10 per cent of the total calorigenic effect of A. Experiments are being carried out at present in an attempt to determine this proportion more accurately, but the results hitherto obtained indicate that this part is insignificant.

A increased the pulse rate by approximately 25 per cent and it is well documented that an infusion of A increases the minute volume of the heart by 50—100 per cent (v. Euler and Liljestrand 1927, Goldenberg *et al.* 1948, Barcroft and Swan 1953). The O_2 consumption of the heart under basal conditions is calculated to be about 11 per cent of the total O_2 consumption (Wade and Bishop 1962). It is obvious that an increased cardiac output will be combined with an increased energy consumption. In studies of the calorigenic effect of isoprenalin in man, a statistically significant correlation was

found between increase in pulse rate and increase in O_2 consumption (Svedmyr and Lundholm unpublished) Exactly how great a proportion of the calorigenic effect of A that can be ascribed to increased cardiac work is difficult to determine however with the data available at present

It has also been found that A infusion in man stimulates the local O_2 consumption in skeletal muscle (Lundholm and Svedmyr 1965) It is possible therefore that the residual effect also comprises direct stimulation of the O_2 consumption of the skeletal muscle

To summarize the experiments performed appear to indicate that stimulation of the FFA and lactate metabolism plays an important role in the calorigenic effect of A Other effects however such as the hyperglycaemic and heart stimulating effects and direct stimulation of the O_2 consumption of skeletal muscle may also be of some importance

CHAPTER VI

The influence of triiodothyronine treatment on some effects of adrenaline and noradrenaline in experiments on man

As mentioned previously Horstman (1934) found that in patients with hyperthyroidism the calorigenic effect of A was potentiated and in hypothyroidism diminished compared with patients with normal thyroid function. In further experiments (study V) the effect of a 30 min infusion of A (0.10 μg/kg/min) and of NA (0.15 μg/kg/min) was first determined in the same way as in study III. The subjects were then given T_3 in a single oral dose of 1 mg and the respective CA infusions were then repeated 2—2.5 days later. T_3 was chosen because its effect begins and disappears more rapidly than that of T_4. The dose of T_3 given induced definite but moderate thyrotoxic symptoms. The basal metabolism increased on the average by 14 per cent in the A group (2.5 days after T_3 medication) and just over 20 per cent in the NA group (2.0 days after T_3 medication) while the pulse increased by 16—17 beats/min and a slight rise in the systolic blood pressure was also noted (Tabel I, study V). A relatively low dose of T_3 was chosen so that the subjects should not be nervous or tense during the experiment and so that the pulse increase and other symptoms should not be too pronounced during the CA infusion.

The effect of adrenaline infusion before and after T_3 medication

After treatment with T_3 the calorigenic effect of A (in ml O_2/kg/min) was potentiated considerably and was practically twice as great as before the treatment (Fig. 9, study V, Table II). The effects of A on the CO_2 elimination and the ventilation were also increased after T_3 medication (Fig. 10).

The effect of A on the lactate concentration in the blood was also increased considerably during the entire infusion period. On the other hand the elevating influence of A on the plasma FFA concentration was somewhat but significantly diminished after T_3 medication. The effect of A on the blood glucose concentration remained unchanged after T_3 had been given (Fig. 10).

Of the circulatory parameters the pulse raising effect of A appeared to be somewhat greater at the end of and after the infusion period following T_3 treatment, while its influence on the blood pressure remained unchanged (Fig. 10, Study V, Table II).

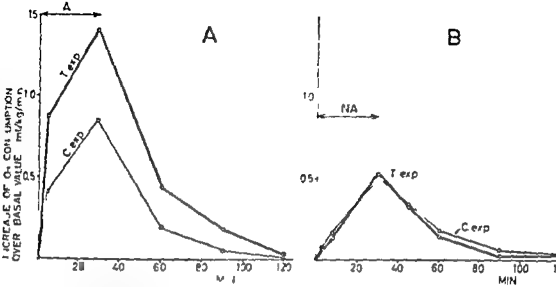

Fig. 9. A. The influence of A infusion (0.10 µg/kg/min for 30 min) on the oxygen consumption of untreated (C exp) and T_3 treated (T exp) people. Mean of 6 experiments.

B. The influence of NA infusion (0.15 µg/kg/min for 30 min) on the oxygen consumption of untreated (C exp) and T_3 treated subjects (T exp). Mean of 5 experiments.

The effect of noradrenaline infusion before and after T_3 medication

The calorigenic effect of NA given for 30 min in a dose of 0.15 µg/kg/min was only about half as great as that of A. The effect was also manifested considerably more slowly than that of A (Fig. 10, study V, Table III). NA had only a negligible effect on the lactate concentration in the blood, while its FFA increasing effect was of the same magnitude and duration as that of A. The hyperglycemic effect of NA was approximately half as great as that of A.

After T_3 medication the calorigenic and other metabolic effects of NA remained completely unchanged, except possibly for a slight potentiation of its hyperlactacidemic action. No influence on the FFA stimulating effect was noted.

The metabolism of L(+) lactate before and after T_3 medication

As in the experiments on the rabbit, thyroid hormone potentiated the calorigenic and hyperlactacidemic effects of A in man. It is obviously possible that the latter effect could have been due to stimulation of the lactic acid production in the tissues or to a decrease in the elimination of lactate from the blood. L(+) lactate (2.3 mg/kg/min for 30 min) was therefore infused

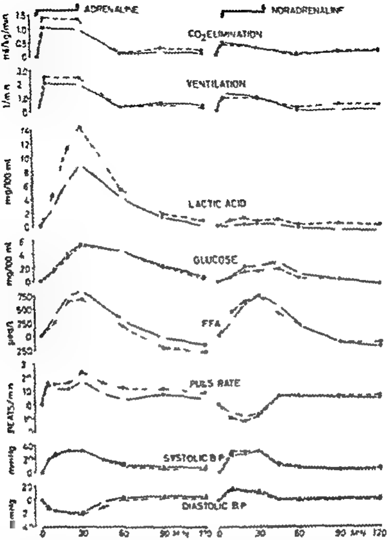

Fig. 10. The influence of A and NA infusions (0.10 resp. 0.15 μg/kg min for 30 min) on some metabolic and circulatory functions in man. Continuous lines — — — — = experiments on untreated people. Broken lines o— — —o— — —o— — —o = experiments on T_3 treated subjects. A experiments: Mean of 6 tests. NA experiments: Mean of 5 tests.

before and after the subject was given 1 mg T_3 perorally. It was found that the lactate increase in the blood was significantly smaller after T_3 medication (Fig. 7, study IV, Table II). The increase in the O_2 consumption, on the other hand, was significantly larger after T_3 had been given.

The results showed that the lactate elimination from the blood was increased after T_3 treatment. The potentiation of the hyperlactacidemic effect of A after

T_3 treatment could therefore be assumed to be due to increased lactic acid production in the tissues

By means of the regression equation which correlated the stimulation of the oxygen consumption (Y) to the increase in the lactate concentration in the blood (X) on infusion of lactate (study IV) before and after T_3 medication the presumable lactate dependent increase in the oxygen consumption following A infusion were calculated This calculated curve is given in Fig 11 The area below this curve was 49 per cent of the total experimentally observed calorigenic effect of A after T_3 medication The corresponding value before administration of T_3 was 40 per cent T_3 medication increased the calorigenic effect of A by 85 per cent It was estimated from Fig 11 that 58 per cent of this increase could be ascribed to potentiation of the lactic acid production and metabolism About half of the potentiating effect of T_3 on the calorigenic action of A could therefore probably be ascribed to increased lactate metabolism

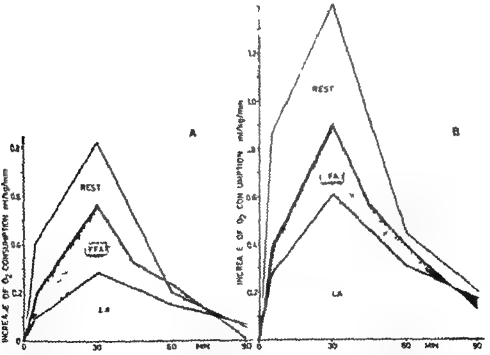

Fig 11 Tentative presentation of the relative importance of lactic acid (L A) and FFA metabolism for the calorigenic effect of adrenaline in untreated (A) and T_3 treated subjects (B) L A increase of oxygen consumption calculated from the relationship between lactic acid content in the blood and stimulation of oxygen consumption as found in study IV FFA part of the calorigenic effect of adrenaline which was blocked by nicotinic acid and attributed to increased FFA metabolism (study III)

The importance of other factors for the calorigenic effect of CA after T_3 medication

An interesting question is that of the role played by FFA mobilization in the potentiating effect of T_3 on the calorigenic action of A. The elevating effect of A on the plasma FFA concentration was slightly smaller after T_3 medication than before. This may either mean that the FFA mobilization was decreased or that the FFA elimination was increased, or again that the FFA mobilization was increased but to a smaller degree than the FFA elimination. In the experiments with NA, however, neither the FFA stimulating nor the calorigenic effects of NA were altered by T_3 medication. Provided that the T_3 medication had not in principle influenced the FFA mobilizing effects of A and NA in different ways, which is not probable, the experiments with NA strongly indicate that the FFA mobilization during the A infusion was not changed either after T_3. The observed effect of A was therefore perhaps due to increased elimination of FFA from the blood.

It seems to be no reason, therefore, for believing that the FFA mobilizing effect of A was of any great importance for the potentiating action of the T_3 medication on the calorigenic effect of A in these experiments.

It is probable that factors other than an increased lactate metabolism played a part in the potentiating effect of T_3 in man. The pulse raising effect of A was potentiated somewhat by the T_3 medication, and it is possible that stimulation of the cardiac output was among the effects of some importance for the increased calorigenic effect of A.

Influence of thyroid hormone treatment on inactivation of infused CA

It is conceivable that the observed potentiation of the calorigenic effect of the infused CA could have been due to inhibited inactivation of A. Circulating CA are inactivated mainly by being taken up by the adrenergic nerves and by enzymatic break down.

The potentiation of the effects of circulating CA may occur by inhibition of the uptake of CA in adrenergic neurons (Whitby, Herting and Axelrod 1960) and by blockade of catechol-O-methyl-transferase (COMT) especially in the liver (Axelrod *et al.* 1958, Axelrod 1959, De Schaepdryver and Kirshner 1961, Carlsson and Waldeck 1963). In both cases this would result in higher concentrations in the blood and at the receptors and in this way the CA effect would be potentiated and prolonged if the tissue receptors remained unchanged (Wylie, Archer and Arnold 1960, Carlsson and Waldeck 1963). A number of investigations have been made in this field (for rev. see Harrison 1964, Rosenberg and Bastomsky 1965). Beavan, Costa and Brodie (1963) found in mice that a T_4 dose that raised the O_2 consumption 75% did not unequivocally affect the amount of endogenous NA in the heart. The content of ^3H-NA taken up was insignificantly reduced and the ^3H-NA turnover in the cardiac muscle was not affected. Wurtman, Kopin and Axelrod (1963), however, observed hyperthrophy and decreased ability of each unit weight of the hyperthyroid rat heart to inactivate ^3H-A by binding; they also found that T_4 treatment neither changed cardiac nor hepatic catechol-O-methyl-transferase activity.

The effect of T_3 treatment on the CA concentration of the blood during infusion of A in man

The CA concentration in the blood before and after T_3 medication was analysed during the A infusion experiment described in study V. The results of these analyses are reported in study VI. As shown (Fig. 12) the plasma A concentration before the A infusion was not affected by the T_3 medication; in the majority of subjects it lay below the determination limit of the method and then rose during the A infusion. The increase during A infusion was the same before as after T_3 medication and after the end of the infusion the A concentration in the plasma decreased rapidly and at a similar rate in both

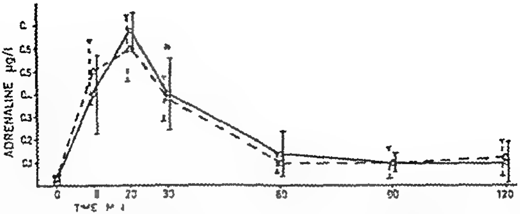

Fig. 12 The adrenaline content of the plasma on i.v. infusion of l adrenaline in a dose of 0.1 µg/kg min during the period 0—30 min. Broken line: Adrenaline content before treatment with triiodothyronine. Continuous line: Adrenaline content 3 days after the administration of 1 mg triiodothyronine by mouth. Mean ± S.E.M. of 5 experiments. * blood sample collected during the 29—31 min

groups. These results contradict the view that the potentiation of certain A effects which were observed in study V after T_3 treatment could be due to influence on the uptake of circulating CA or to inhibition of those enzymes (chiefly COMT) that are of importance in the break down of circulating CA.

The effect of T_4 treatment on the metabolism of tritium labelled catecholamines in the rat

Further support for the above results was obtained in study VII. In this study an investigation was made of the effect of T_4 given in a dose that increased the oxygen consumption about 30 per cent on the concentration of ^{3}H-NA, ^{3}H-A and their metabolites ^{3}H-normetanephrine (^{3}H-NM) and ^{3}H-metanephrine (^{3}H-M) in cardiac muscle, femoral muscle, diaphragm and blood in the rat after infusion of the respective ^{3}H-CA in low dosage. The concentration of ^{3}H-A in the cardiac muscle was the same in both the control group and the T_4 treated group (Fig. 13). Neither was any effect of T_4 treatment shown on the concentration of ^{3}H-A in femoral muscle, diaphragm or blood plasma. The ^{3}H-M concentration remained unchanged by T_4 treatment. In experiments with infusion of ^{3}H-NA the standard error of the mean was greater, possibly due to the fact that the experiments were extended over a long period. No significant effect of T_4 was observed in these experiments either (study VII, Fig. 2). To summarize, studies VI and VII indicate that the potentiation of certain effects of CA after administration of thyroid hormones appears to be due to changes in the receptor cell rather than to reduced uptake or break down of CA.

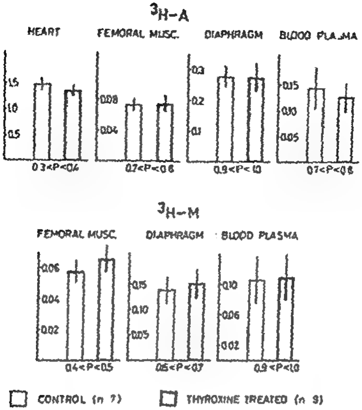

Fig 13 The effect of thyroxine treatment on the turnover of infused tritiumlabelled adrenaline in the rat

^{3}H A was infused in a total dose of 1 μg/kg for 30 minutes Thyroxine 0 10 μg/animal day for [illegible] days Content of ^{3}H A and ^{3}H M in μg/g tissue immediately after the end of the infusion Mean ± S E M

CHAPTER VIII

General discussion

It is evident from the experiments described that the potentiating effect of thyroid hormones on the calorigenic action of A may probably be ascribed to a large part to an increase of the lactate producing effect of A in the tissues. This increase does not appear to be explainable by inhibition of the inactivation of infused A and it would seem rather to be due to influence on the effector cell.

The mechanisms whereby CA appear to stimulate the O_2 consumption is illustrated by Fig. 14.

Of interest for this discussion are the strong indications that cyclic 3—5 AMP apart from mediating the phosphorylase activating and thereby the glycogenolytic hyperglycemic and hyperlactacidemic effects of CA also mediates their FFA mobilizing and heart stimulating effects (for review see Haugaard and Hess 1965). This points to the possibility that cyclic 3—5 AMP is of a more general importance for the mediation of some of the effects of CA on different biochemical and pharmacological reactions. This has been discussed in detail by Lundholm, Mohme Lundholm and Svedmyr (1966).

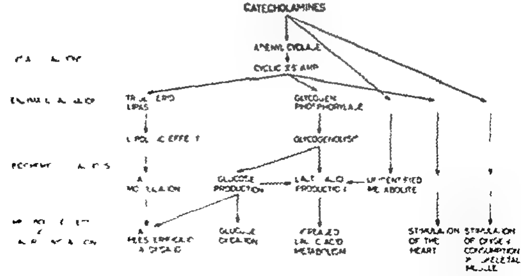

Fig. 14. Different mechanisms which may be of importance for the calorigenic action of the catecholamines. For further information see the text.

The most simple explanation for the potentiation of the effects of A by thyroid hormones would therefore be that they increase A's stimulation of the production of cyclic 3—5 AMP in the tissues. Owing to the difficulties involved in determining the concentration of cyclic 3—5 AMP in the tissues, however, it has not been possible up to the present time to prove this hypothesis by direct experiments, and the problem has had to be approached in other ways.

In consideration of the factors discussed above, I carried out a study on the way in which T_4 medication influences the glycogenolytic and lactic acid producing effects of A in skeletal muscle. The object chosen for this study was rat diaphragm, in which different investigators (Riesser 1947, Walaas 1950, 1955) had previously demonstrated that A had these effects.

These experiments indicated that T_4 medication selectively potentiated the lactate producing effect of A, while the glycogenolytic effect remained unchanged. After thyroidectomy, A showed neither any glycogenolytic nor hyperlactacidemic action in the diaphragm (Svedmyr 1965a). T_4 treatment did not alter the phosphorylase activating effect of A in this muscle (Svedmyr 1965b), an observation that was also made by Hess and Shanfeld (1965) in cardiac muscle. These results are in agreement with the finding that the glycogenolytic effect of A was not potentiated in diaphragm from T_4 treated rats. The source of origin of the increased lactate production is not as yet localized — hexose phosphates and glycerol can probably be excluded (Svedmyr, unpublished). It is possible that in the diaphragms from T_4 treated animals A stimulated the glyconeogenesis. It is of interest that Exton and Park (1965) recently showed that cyclic 3—5 AMP and hormones that stimulated its production (glucagon and A) increased the glyconeogenesis in isolated rat liver.

The question of whether T_4 influenced the A effect by sensitizing the adrenergic receptor, or by influencing the reactions that are provoked by the receptor cannot be definitely answered as long as the point of attack of the potentiating action of thyroid hormones on the lactate stimulating effect of A is not localized. There is some evidence to support the latter view, however. In experiments on the rabbit (study I) it was found that T_4 treatment significantly increased the effect of a relatively large dose of A (0.5 $\mu g/kg/min$) but not with certainty a smaller dose (0.05 $\mu g/kg/min$). If T_4 had sensitized the adrenergic receptors it would have been expected that the effect of the smaller dose would have been increased at least as much as that of the larger dose. In experiments on the rat diaphragm, A was also used in a concentration that could be expected to have a maximal glycogenolytic and lactate producing effect in untreated animals. In spite of this, the T_4 treatment increased the lactate producing action of A. These findings appear to support the hypo-

thesis that T_4 medication influenced the A effect by action on the reactions that are provoked by the receptor It is of interest in this connection that the effect of cyclic 3—5 AMP on the effector organ can also be modified by certain drugs Northrop and Park (1964) thus found that the hyperglycemic effect induced by cyclic 3—5 AMP *in vivo* in the rat was potentiated by theophylline and inhibited by DHE but was not influenced by adrenergic β blocking agents The questions above are the subject of further studies

General summary

It is well documented that thyroid hormones can potentiate certain of the pharmacological effects of CA The aim of the present investigation was to attempt to determine 1) whether thyroid hormones influence the calorigenic effect of CA in the rabbit and in man 2) which mechanisms were of importance for mainly, the calorigenic effect of A 3) the way in which thyroid hormones influence these mechanisms the following questions being studied in particular a Do thyroid hormones influence the inactivation of infused CA so that higher concentration affects the adrenergic receptors over a longer period? b Do thyroid hormones sensitize the adrenergic receptors? or c Are those biochemical processes affected which may conceivably be stimulated by the adrenergic receptors?

In experiments on un anaesthetized rabbits (study I) A increased the oxygen consumption and also the blood lactate and glucose concentrations considerably The plasma FFA concentration rose initially during the infusion but then decreased to below the basal value despite further infusion The calorigenic effect of NA in a similar dose was only about 1/3 that of A NA induced only a very slight increase in the lactate concentration in the blood but its effect on the FFA concentration was of equal magnitude to that of A though of longer duration The hyperglycemic effect of NA was about 1/6 that of A

After medication with T_4 (1 mg/day subcutaneously for 7 days) the basal oxygen consumption of the animals increased by 45—65 per cent After T_4 treatment the calorigenic and hyperlactacidemic effects of A were significantly increased T_4 treatment did not alter the calorigenic or other metabolic effects of NA The effect of an A dose ten times smaller was not potentiated with certainty by T_4 treatment Four to six weeks after thyroidectomy when the basal oxygen consumption of the rabbits was decreased by 20 per cent the calorigenic and lactate increasing effects of A were significantly diminished

On infusion of L(+) lactate (11 5 mg/kg/min) in untreated animals the blood lactate concentration rose to an equal extent as after A infusion In T_4 treated rabbits a similar lactate infusion gave a significantly lower blood lactate concentration while in thyroidectomized animals this was of approximately the same order of size as in the untreated animals (study II) The lactate

elimination was thus increased after the T_4 treatment and it is evident from this that T_4 potentiated the lactate producing effect of A in the tissues but that this was reduced after thyroidectomy.

The lactate infusion stimulated the O_2 consumption in the rabbit considerably. On correlating the increase in oxygen consumption with the increase in blood lactate concentration the correlation coefficients were significant in the different groups and by means of the regression equations obtained that proportion of the O_2 consumption increase that probably resulted from the increase by A of the blood lactate concentration was calculated under the assumption that this relationship was the same during a lactate as during an A infusion. It was thus estimated that a large proportion of the calorigenic effect of A was due to increased lactate metabolism both in the untreated, thyroidectomized and thyroxine treated rabbits. The influence of thyroid hormones on the calorigenic effect of A in rabbits may therefore be ascribed at least in part to an increased lactic acid production and metabolism.

In experiments on man (study III) the importance of the FFA mobilizing and hyperlactacidemic effects of A for its calorigenic effect was studied. When the FFA mobilizing effect of A was blocked selectively with nicotinic acid the total calorigenic effect was reduced by about 30 per cent. Infusion of 2.3 mg/kg/min L(+) lactate raised the lactate concentration of the blood to approximately the same extent as did A in a dose of 0.10 μg/kg/min. From the relation between the lactate increase in the blood and the increase in oxygen consumption the magnitude of the effect that could be expected to be due to the hyperlactacidemic effect of A was calculated as in the rabbit experiments. This was estimated to be about 35—40 per cent of the total calorigenic effect of A.

Different alternatives are discussed for the way in which an FFA and lactate increase can be considered to stimulate the oxygen consumption. Since the available experimental data are partly controversial and partly insufficient no definitive answer can be given to this question. There seems to be some evidence, however, that the increase in the O_2 consumption is mainly induced by the different resynthesization processes which follow an increase in the concentrations of lactate and FFA in the blood. It is considered that the liver plays an important role in these processes.

It seemed probable that other mechanisms than the lactate and FFA mobilization were of importance for the calorigenic effect of A in man. Among these, the heart stimulating and hyperglycemic effects of A are discussed and also a direct stimulatory effect of A on the oxygen consumption in skeletal muscle. The relative contribution to and the importance of these effects for the total calorigenic effect of A are not yet determined, however.

Triiodothyronine (T_3) in an oral dose of 1 mg increased the oxygen consumption in man by 14—22 per cent after 2—2 5 days Other thyrotoxic symptoms such as increased pulse rate slight tremor and nervousness also occurred The calorigenic effect of A was approximately doubled and the elevating effect of A on the blood lactate concentration was also increased The FFA mobilizing effect of A was slightly reduced while its hyperglycemic effect was unchanged The stimulatory effect of A on the respiration and CO_2 production was potentiated constantly by the T_3 treatment and the pulse raising effect was also slightly increased

The calorigenic effect of NA in untreated subjects was approximately 40 per cent that of A NA had a very weak lactate increasing action while the FFA increasing effect was similar in magnitude and duration to that of A The hyperglycemic effect of NA was about half as strong as that of A

T_3 medication did not potentiate the calorigenic or FFA mobilizing effects of NA and neither did it have any significant influence on the other metabolic effects of NA

On infusion of L(+) lactate (2 3 mg/kg/min for 30 min) in T_3 treated subjects the blood lactate concentration rose to a significantly smaller extent than before the administration of T_3 while on the other hand there was greater stimulation of the oxygen consumption From the correlation between the lactate increase in the blood and the stimulation of the oxygen consumption in the T_3 treated persons it was calculated that about 50 per cent of the calorigenic effect of A could be ascribed to an increased lactate metabolism T_3 increased the calorigenic effect of A by 85 per cent and about half of this increase could be assumed to be due to an increase in lactate production and metabolism

The question of whether thyroid hormone treatment affected the inactivation of infused CA was studied in experiments on the rat No influence of T_4 treatment on the uptake of infused ^{3}H labelled A or NA in different organs was observed Neither was the inactivation of CA via COMT affected by T_4 medication On infusion of A in man medication with T_3 had no influence on the plasma A concentration before during or after the infusion It was therefore unlikely that thyroid hormones increased the effect of A by inhibiting its inactivation

In experiments on the rabbit T_4 medication potentiated the effect of an A dose which had an almost maximal lactate producing action The effect of a dose ten times lower seemed not to be potentiated It was therefore not probable that the T_4 had sensitized the adrenergic receptors for A The point of attack was instead assumed to be situated below the primary reaction between CA and the receptor

On the basis of *in vitro* experiments on rat diaphragm some alternatives are discussed for the way in which thyroid hormones can conceivably potentiate the lactate producing action of A. It does not appear that the point of attack of thyroid hormones can be localized to any of the more well known effects of A in the rat diaphragm such as its glycogenolytic effect or phosphorylase activation.

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STUDIES OF ACUTE RESPIRATORY ACID-BASE CHANGES IN BRAIN TISSUE AND CEREBROSPINAL FLUID

An approach to the acid base metabolism of the brain
in vivo

BY

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FROM THE DEPARTMENT OF NEUROSURGERY, UNIVERSITY OF LUND, AND THE NEUROSURGICAL RESEARCH LABORATORY, THE HOSPITAL OF LUND, SWEDEN

STUDIES OF ACUTE RESPIRATORY ACID-BASE CHANGES IN BRAIN TISSUE AND CEREBROSPINAL FLUID

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Printed in Sweden

MALMÖ [illegible]

SYDSVENSKA DAGBLADETS AKTIEBOLAG

Återkomst

Barnen drar mig i rockens skört
— Kommer du hem på din ålders år?
Vad för en tävlingskamp har du fört?
Var har du vunnit ditt vita hår?

Tu Mu
803—852 e. Kr.

The present thesis is based on the following papers

I PONTÉN U and B K SIESJÖ A method for the determination of the total carbon dioxide content of frozen tissues
Acta physiol scand 1964 60 297—308

II PONTÉN U and B K SIESJÖ Acid labile carbon dioxide of rat brain after freezing the tissue *in situ*
Acta physiol scand 1964 60 309—317

III PONTÉN U and B K SIESJÖ Gradients of CO tension in the brain
Acta physiol scand *1966 In press*

IV PONTÉN U Acid base changes in rat brain tissue during acute respiratory acidosis and baseosis
Acta physiol scand 1966 In press

V PONTÉN U Consecutive acid base changes in blood brain tissue and cerebrospinal fluid during respiratory acidosis and baseosis
Acta neurol scand 1966 In press

The above papers will be referred to as *Paper I V*

Since it is of vital importance for the organism to keep the acidity of its different fluid phases within narrow limits much interest has been directed towards measuring deviations from the normal acid base balance in blood and other tissues. Measurements on blood will to some extent provide knowledge of the acid base metabolism of the organism as a whole. It has been recognized that deviations from the normal in the blood acid base parameters are usually accompanied by an altered acid base metabolism of the individual tissues, but only recently have systematic studies of the acid base metabolism of tissues been performed. The reason for this is the theoretical conceptual and experimental difficulties encountered when the acid base relations of multi compartment tissue systems are considered.

ACID BASE RELATIONS IN BLOOD AND OTHER HOMOGENEOUS LIQUID PHASES

The Henderson Hasselbalch equation

The acid base metabolism of a system is usually approached by studying one of its buffer systems. The most important biological buffer is the bicarbonate/carbonic acid buffer system. Its prominence is due to its existance in high concentrations in body fluids and tissues and that its acid is volatile and regulated by pulmonary ventilation. The relation between the components of the buffer system is defined by the well known Henderson—Hasselbalch equation

$$pH = pK + \log \frac{[HCO_3^-]}{[CO_2] + [H_2CO_3]} \qquad (1)$$

$$= pK'' + \log \frac{[TCO_2] - [PCO_2 \; S_1]}{[PCO_2 \; S_2]} \qquad (2)$$

$$= pK'' + \log \; BR \qquad (3)$$

In equation (1) pK is the first apparent ionization constant of carbonic acid, HCO_3 the bicarbonate concentration and H_2CO_3 the concentration of dissolved carbonic acid. By convention the acid concentration is set equal to the sum of the dissolved CO_2 and the carbonic acid (CO_2+H_2CO_3). Since the concentration of H_2CO_3 can be regarded as negligible in comparison with the concentration of dissolved carbon dioxide the acid concentration is expressed as the product of the carbon dioxide tension and a solubility coefficient (S_1 or S_2). This convention

requires the true apparent ionization constant of carbonic acid (pK) to be replaced by another constant (pK) Equation (2) indicates the usual calculation of the bicarbonate concentration as the difference between the total acid labile carbon dioxide content (T_{CO_2}) and the amount of CO_2 dissolved (P_{CO_2} S_1)

The Henderson—Hasselbalch equation has been derived for and is strictly applicable to only a one compartment system The constant S must be determined after acidification and pK by determining pH and the bicarbonate concentration at known carbon dioxide tensions Both these constants must be determined for every new system considered When the two constants are known determinations of two of the experimental variables will suffice to define the third P_{CO_2} and pH are easily determined with modern techniques and the equation is essentially used to derive actual and standard bicarbonate values

Evaluation of acid-base shifts

A change in pH can be regarded as the resultant of two components viz a respiratory and a nonrespiratory (metabolic) shift These two components are independent if physicochemical buffering only is considered (i.e *in vitro*) The respiratory shift is fully defined by changes in the carbon dioxide tension The nonrespiratory shift is partly determined by changes in the bicarbonate concentration at a defined carbon dioxide tension i e by changes in standard bicarbonate (JORGENSEN and ASTRUP 1957) The standard bicarbonate change will reflect the buffering of strong acid or base by the CO buffer system which in blood accounts for about 80 per cent of the total buffering against nonvolatile acids or bases

To account for the entire nonrespiratory acid base shift it is necessary to titrate the system with strong acid and base or to know the amounts of available nonbicarbonate buffer groups in the system In blood these groups almost exclusively consist of the histidin residues of the haemoglobin molecules (EDSALL and WYMAN 1958) The nonbicarbonate buffer groups will be responsible for about 20 per cent of the buffering against strong acid or base but they determine the whole buffer capacity against carbon dioxide defined as

$$\beta_{CO_2} = \frac{\Delta \log P_{CO_2}}{\Delta \, pH} \tag{4}$$

$$= \frac{\Delta \log P_{CO_2}}{\Delta \log \dfrac{[HCO_3^-]}{P_{CO_2} \, S_2]}} = \frac{\Delta \log P_{CO_2}}{\Delta \log BR} \tag{5}$$

Equation (4) (SIESJÖ 1962 d) will define the slope of the linear relation that will be obtained if a blood sample is equilibrated *in vitro* with different carbon dioxide tensions and if the pH values measured in the plasma are plotted against the carbon dioxide tension in a log P_{CO_2} / pH diagram (BREWIN *et al* 1955 ASTRUP 1956) Equation (5) will apply to tissue studies (see below) but is defined here for the sake of convenience

The nonrespiratory acid base shifts can be expressed either as the change in buffer base (equal to the sum of the buffer anions SINGER and HASTINGS 1948) or as the base excess values i e deviations from the normal buffer base Base excess values can be determined by titrating blood samples *in vitro* at a constant carbon dioxide tension with strong acid or base to a normal pH (SIGGAARD—ANDERSEN 1963)

In determining base excess values the blood is titrated *in vitro* with different CO_2 tensions and the resulting curve then expresses the *in vitro* buffer capacity against CO This buffer curve differs from that which can be determined *in vivo* by equilibrating *the intact organism with different CO tensions before* the blood is sampled (SCHWARTZ and RELMAN 1963) The slope of the *in vivo* curve (CO_2 buffer capacity) changes with time In the acute stage (COHEN *et al* 1964) the slope is less than the *in vitro* slope, reflecting the fact that there is an exchange of bicarbonate ions between blood and the poorly buffered extra cellular fluid In the chronic stage (SCHWARTZ *et al* 1965) the curve has a steeper slope than the *in vivo* curve since renal and other mechanisms have given rise to a retention of bicarbonate ions

In the physiological approach to the acid base metabolism advocated by SCHWARTZ and RELMAN (1963) the nonrespiratory component is evaluated from the CO tension and the actual bicarbonate concentration Thus the difference between the actual bicarbonate concentration and that predicted from the *in vivo* titration curve is a measure of the nonrespiratory component This change in bicar bonate lacks the precision of the base excess term which is defined for the blood only but on the other hand it accounts better for the complex pattern of mechanisms occuring *in vivo* Since tissues like the brain cannot succesfully be equilibrated *in vitro* the physiological approach is the only one applicable

Experimental studies of the acid base metabolism of tissues like the brain have certain advantages Thus the total amount of strong acid or base added to the organism cannot be determined from measurements on one compartment only viz the blood but must be assessed from total body balance studies When the brain is studied the bicarbonate content of the whole tissue can be determined and if a reasonable estimate for the nonbicarbonate buffer concentration can be obtained base excess values can be defined This is obviously a way of determining net transport between the tissue and the blood

TISSUE ACID BASE RELATIONS AND THE HENDERSON-HASSELBALCH EQUATION

Difficulties arise in the study of tissue acid base parameters mainly because a tissue is a multi compartment system The extent to which the terms of the Henderson—Hasselbalch equation are applicable to such a system will now be considered

The actual pH

The mean hydrogen ion activity can be determined directly if micro pH electrodes are introduced into single cells (CALDWELL 1958, KOSTYUK and SOROKINA 1960) This procedure only gives information regarding the pH of the cytoplasmic phase of large cells but not of minor tissue compartments such as surface phases mito chondria etc The technique is hardly applicable to tissues such as the brain in which the cells are small and difficult to penetrate even with ordinary ultra micro electrodes

While there are practical difficulties in measuring the cytoplasmic pH of some cells there are also theoretical objections to the calculation of intracellular pH from the distribution of weak acids such as carbonic acid or DMO the latter introduced by WADDELL and BUTLER (1959) for tissue acid base studies and used by ROOS (1965) in a study on intracellular pH of cat brains This method measures the mean concentration of the buffer anion which has an uncertain relation to the mean hydrogen ion concentration or activity (for discussion see Paper IV) Moreover the pH term is hardly applicable to multi compartment systems (SIESJO and PONTEN 1966 a)

The apparent ionization constant of carbonic acid (pK)

The pK is a composite term which besides the equilibrium constant includes the activity coefficients of the conjugate acid base pair (CO H_2CO_3 HCO_3) If pK is to be determined for a tissue system the tissue must be disrupted to yield a continuous liquid phase and it is by no means certain that the pK of this artificial system is a meaningful reflection of the equilibrium constant and the activity coefficients in the discrete compartments of the intact tissue (SIESJO 1962 a) The pK term will obviously suffer the same restrictions and uncertainties as the pH in a multi compartment system

The solubility coefficients

In determining the bicarbonate concentration from the relation $HCO_3 = TCO_2 - PCO_2$ S_1 the solubility coefficient must express the total amount of dissolved carbonic acid irrespective of the tissue phase Such a coefficient can only be determined if the tissue is acidified and thus only on a disintegrated tissue

(SIESJÖ 1962 a) The application of the solubility coefficient to the intact tissue will depend on the validity of an extrapolation from homogenates However since there is a linear relation between the solubility coefficient and the tissue concentration in the homogenates there is no reason to assume that the extrapolated value will deviate appreciably from the true solubility of the intact tissue (van SLYKE *et al* 1928 SIESJO 1962 a) A small error in the solubility factor (S_1) will also be reasonably insignificant Thus for a total carbon dioxide content of about 14 mMoles/kg a 10 cent error in S_1 will cause an error in the bicarbonate value of less than 1 per cent

It is generally assumed that bicarbonate ions are distributed only in the water phase of the tissue *Furthermore in deriving the buffer ratio (buffer ratio* = BR see equation 3) the solubility coefficient must be assumed for it cannot be determined by direct experiments (SIESJO 1962 c) The numerical value will critically influence the buffer ratio and thus any calculated pH A 10 per cent error in S will cause about the same error in the buffer ratio and an error *in the calculated pH of 0 04 units The solubility coefficient for carbonic acid* in physiological saline was chosen as a reasonable estimate of S (SIESJO 1962 c)

The total carbon dioxide content of the tissue (T_{CO_2})

The total content of acid labile carbon dioxide compounds in a tissue will include molecular CO H CO₃ HCO₃ CO₃ and any carbamino CO compounds Neither H CO₃ CO₃ nor carbamino CO are present in brain tissue in significant amounts (see SIESJO 1962 a and c) Thus the relation $T_{CO_2} - P_{CO_2}\ S_1$ will express the bicarbonate content of the tissue For a one compartment system a content per unit weight denotes a concentration In a multi compartment system however a total content per unit weight should not be equated with a mean concentration since the definition of such a term will offer no additional knowledge about the system but might easily lead to a misinterpretation of the data This distinction (total content per unit weight versus mean concentration) has not been rigorously made in the preceeding papers of this thesis but the interpretation of data has not been extended over the limits of the concept of a content per unit weight

The carbon dioxide tension

The tissue carbon dioxide tension is an ambiguous term since there will be various carbon dioxide tensions within the tissue The difference between the highest and the lowest carbon dioxide tension in a tissue will be approximately equal to the arteriovenous carbon dioxide tension difference because of the rapid diffusion of carbon dioxide in the tissue (SIESJO and THEWS 1962) However it is possible both to define theoretically a mean tissue carbon dioxide tension (P_{tCO_2}) and to verify its value directly (Paper III)

It might be said in conclusion that there are serious objections to the application

of the Henderson—Hasselbalch equation to tissue systems the chief of which is that mean activities and mean concentrations of ions cannot be defined in a *meaningful way in multi compartment systems* The pH term should be avoided since the hydrogen ion content can neither be measured nor calculated The term buffer ratio is less ambiguous than pH but its use in the tissue signifies the relation between two contents and its interpretation as a resultant of respiratory and nonrespiratory acid base shifts must be dealt with cautiously Moreover its numerical value is critically influenced by the assumption regarding the solubility *coefficient* (S)

Although *the difficulties connected with the use of terms like pH pK* and log BR in tissues preclude the application of the Henderson—Hasselbalch equation there are two factors which still have a quantitative meaning *viz* the bicarbonate content of the tissue and the mean tissue carbon dioxide tension Both these parameters can be directly measured or derived and the assumptions involved cannot critically influence the values obtained Moreover a quantitative acid base *system can be established with these two parameters if the in vivo* buffer capacity of the tissue *is known*

THE PRESENT APPROACH TO THE ACID BASE METABOLISM OF BRAIN TISSUE

This thesis is concerned with some aspects of a simplified approach to the acid base metabolism of brain tissue These aspects will be discussed under the following headings

1 The total carbon dioxide content (Paper I II and IV)
2 The mean tissue carbon dioxide tension (Paper III)
3 The *in vivo* carbon dioxide binding curve of brain tissue during acute respiratory acid base shifts (Paper IV)
4 An equivalent buffer system (Paper IV)
5 *Intercompartmental differences in the acid base metabolism of brain tissue* (Paper III and V)

The total carbon dioxide content of brain tissue

It has been found that the total carbon dioxide content is sensitive to *post mortem* changes *In situ* freezing of the brain is thus obligatory if reliable total carbon dioxide values are to be obtained A technique is described for freezing the brain of rats *in situ* with liquid nitrogen The technique seems to give an adequate *in vivo* fixation of the tissue as judged from the lactate contents of the tissue *A method is described for the determination of the total acid labile* carbon dioxide of the frozen samples The method utilizes a single diffusion step The carbon dioxide liberated by acidification of the sample is absorbed into barium hydroxide and the amount of carbon dioxide is subsequently determined by titration The coefficient of variation of the method is 0 9 per cent

The mean carbon dioxide tension of brain tissue

The mean tissue CO tension can be calculated to exceed the arithmetic mean of the arterial and cerebral venous carbon dioxide tensions by 0 5 — 1 0 mm Hg (GLEICHMANN *et al* 1962) The carbon dioxide tension which could be measured in the cerebrospinal fluid (CSF) and on the cortical surface coincided with the theoretically derived value The arterial carbon dioxide tension and the CO tensions measured on the cortical surface in the CSF and in the blood from the superior sagittal sinus were so consistent that evaluation of the mean tissue carbon dioxide tension from the measured arterial tension was justified This conferred a definite experimental advantage since it was found that the carbon dioxide tension gradients in the brain were significantly influenced by a craniotomy The coefficient of variation of the arterial carbon dioxide tension determinations can be estimated to less than 2 per cent

The CO_2 binding curve in vivo

The *in vivo* carbon dioxide binding curve in acute respiratory acidosis and baseosis is a measure of the buffer capacity of the tissue against carbon dioxide This buffer capacity is a summation of the effects of several homeostatic mechanisms Some of these mechanisms can be assumed to be complete within a few min viz true physicochemical buffering and metabolic readjustments causing changes in the levels of nonvolatile acids (e g lactate) or available buffer groups such as various phosphates However there are other mechanisms probably involving active and passive transport of ions which apparently lead to slow bicarbonate changes in spite of a constant CO tension (see NICHOLS 1958 SIESJÖ 1965)

The buffer capacity should preferrably be expressed in terms of the measured quantities i e as the change in bicarbonate content per unit change in tissue carbon dioxide tension However, this ratio will decrease continously with increasing carbon dioxide tension A more constant buffer capacity at varying CO tensions will be obtained if it is defined as in equation (5) above

Approximate comparisons of nonrespiratory acid base shifts between different experimental groups can be obtained from the buffer curve in the linear HCO_3/P_tCO_2 diagram The curve defines the respiratory component of the acid base shifts Nonrespiratory acid base shifts appear as almost parallel shifts of the curve Different experimental groups can be compared by displacing the experimental points in a parallel fashion along the buffer curve to a reference carbon dioxide tension (standard bicarbonate contents) This procedure will be fairly exact in normo- and hypercapnia if the differences in carbon dioxide tension are moderate The steep regression of bicarbonate on carbon dioxide tension in hypocapnia diminishes the accuracy of the procedure

The equivalent buffer system

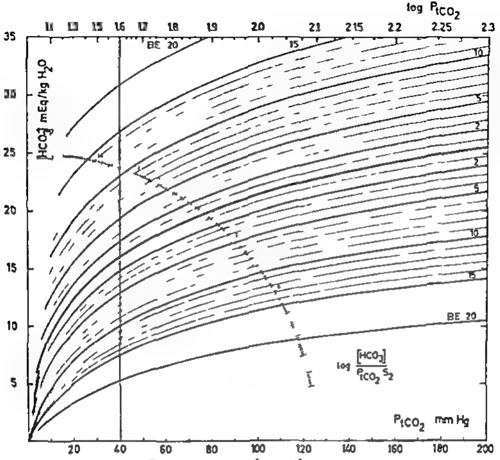
Bicarbonate values do not account for the total amount of nonvolatile acid or base added to a system in nonrespiratory acid base changes (see above) Information on the nonbicarbonate buffers of the brain tissue is incomplete, but some will be provided by the present CO binding curve Thus if an equivalent buffer system is set up which has the same bicarbonate content and the same buffer capacity to carbon dioxide as the experimental curve it will be possible to give a full description of the nonrespiratory acid base changes However the calculation will be based on the following assumptions and simplifications

1 The tissue is treated as a one compartment system
2 Any modification of the acid base state unaccomplished by the bicarbonate/carbonic acid buffer system is ascribed to a single hypothetical buffer (A/HA) with an optimal pk (i e pk equals the mean pH' of the system)
3 The solubility coefficient for the water phase of the tissue is assumed to be the same as for physiological saline
4 pk was assumed to be 6 8 Errors in the second decimal place of this factor will be quite insignificant in these calculations
5 The contents of (H⁺) (CO 3) and (OH) of the tissue were disregarded in the electrical neutrality equation Within the pH limits 5 0 — 7 8 the error caused by these assumptions will be below 2 per cent

Calculations from the theoretical buffer system and from the experimental data showed that about 70 per cent of the tissue buffering against nonvolatile acids was accomplished by the bicarbonate/carbonic acid buffer system A value of 35 mMoles/kg tissue H O for the concentration of the nonbicarbonate buffers was derived Since the experimental carbon dioxide binding curve not only reflects physicochemical buffering but also other mechanisms (see above) the derived nonbicarbonate buffer concentration will probably be slightly high However if the pk values of the *real* buffer groups in the tissue deviate from the optimal pk used in the calculation a higher total nonbicarbonate buffer concentration than that calculated is *required* to account for the experimentally determined buffer capacity of the tissue These two errors will thus tend to cancel each other but their magnitudes are difficult to assess

A buffer base value was derived This is a value for the total amount of buffer anions in the rat brain tissue If there are other mechanisms than physicochemical buffering which contribute to the tissue bicarbonate changes (such as changes in the glycolysis or ion transport) the buffer base will be overestimated The true figure will then be lower than the derived value (36 mEq/kg tissue water)

The base excess values which are identical with the changes in buffer base and quantify the nonrespiratory acid base shifts will be less critically dependent



Curve nomogram for rat brain tissue

on the inherent assumptions of the calculations than will the absolute buffer base values

The Figure illustrates a curve nomogram showing the relation between the tissue carbon dioxide tension and the tissue bicarbonate content along linear coordinates The nomogram was derived from the equivalent buffer system and from the *in vivo* CO_2 binding curve (thick line) obtained in rats anaesthetized with barbiturates and exposed to respiratory acidosis and baseosis for 30 min (Paper IV) The thin curves in the nomogram denote the calculated base excess values Log buffer ratio values for any point in the diagram can be obtained by drawing straight lines from the origin through the experimental points The values are then read on the circular log BR scale at the points of intersection with the straight lines

Acid base parameters in various compartments of the brain

The CO_2 binding curve reflects both intra and extracellular events An adequate differentiation of such events is impossible since neither the size nor the composition of the extracellular phase are known It is often assumed that the cerebrospinal fluid (CSF) reflects the composition of the interstitial fluid of the tissue but there is considerable evidence which indicates that the bicarbonate concentration varies between different parts of the CSF system and also between the CSF and the interstitial fluid (AMES *et al* 1964 PAPPENHEIMER *et al* 1965) While these differences remain unquantified and the size of the combined extracellular space inaccurately known a calculation of extracellular and intracellular acid base events will be a crude approximation However simultaneous determinations of acid base events in arterial blood in total brain tissue and in CSF will yield information not only on the approximate bicarbonate content of the various phases but also on the changes brought about by alterations in carbon dioxide tension as well as on the time course of these changes These studies have shown that the magnitude of the bicarbonate changes is far larger in the CSF than in the total tissue or in the blood They give additional support to the view that bicarbonate changes in the CSF do not passively reflect CO buffering in the blood or in the tissue (PAPPENHEIMER *et al* 1965 SEVERINGHAUS *et al* 1963)

Although it is impossible at present to differentiate between extra and intracellular acid base changes studies of the acid base metabolism of the total tissue phase can provide information regarding the net transport of bicarbonate or hydrogen ions between the blood and tissue and also regarding net accumulation of nonvolatile acids and bases in any of the tissue compartments Simultaneous studies on the total tissue phase and on accessible compartments of the brain tissue (at present the CSF) can also give information on the source of the acid base changes observed However rigid interpretation of such data will by necessity be hampered until more information is obtained regarding the size of the extracellular space

SUMMARY

The present thesis has examined the problems of method involved in the analysis of acid base changes in brain tissue. Optimal methods for the determination of the carbon dioxide content of the brain tissue in vivo were developed. Relations were obtained to enable a mean tissue carbon dioxide tensions in the brain to be calculated from the carbon dioxide tension measured in the arterial plasma. A carbon dioxide binding curve was determined in vivo on anaesthetized rats in acute experiments. This curve quantifies the acute respiratory acid base changes and thus permits a differentiation between acute respiratory and nonrespiratory acid base changes in the rat brain. Quantitative measures of the nonrespiratory acid base shifts were obtained from the consideration of a theoretical equivalent buffer system. Direct simultaneous determinations of acid base parameters in plasma, cerebrospinal fluid and total brain revealed large differences between the compartments both in the magnitude and the time course of the changes. The methodological and conceptual problems involved in studies of the acid base metabolism of a tissue are discussed.

Acknowledgements

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I Preface

A number of earlier papers from this laboratory have dealt with the influence of gravitational and inertial forces on respiratory and circulatory dynamics The present work partly being an extension of these investigations was carried out during the years 1965—1967

I wish to express my appreciation to Professor Hilding Bjurstedt Head of the Department of Aviation Medicine whose generous support has meant everything for carrying through this work I am likewise greatly indebted to Professor Carl Magnus Hesser Head of the Department of Naval Medicine for stimulating advice and constructive criticism

To Dr Ove Wigertz I am indebted for his kind interest and cooperation in the utilization of electronic data processing for facilitation of computations involved in dye dilution techniques My thanks are also due to Drs Georg Matell and Per Olof Barr for valuable discussions

This work could not have been performed without the skilful contributions of the entire technical staff I want especially to express my thanks to Christina Borgh Irene Unander Scharin Karin Vasser Carl Huldt, Bertil Lindborg Bertil Lundin and Axel Lonn

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Stockholm, March 1967
GUNNAR ROSENHAMER

List of Symbols and Abbrevations

dless quantity in the physiological reaction nomenclature (Dixon and Pat er) Cl k Hards and Crosbie 1961 Cauer and Zuidema 1961) a ratio which ex ight force) as well as acceleration in multiples of standard weight (W_0) and eleration of gravity (g_0) In the present study G will be regarded and treated fr m the force side of its dual aspect For example a force of 3 G units the t centrifugal (inertial) and gravitational force components indicates a force f r W = body mass × g_0 G possesses the same vector property of direction as e h t connotes

ording to the Atlantic City Convention (Pappenheimer *et al*

r ls

lume in general
ar per unit time
in general including partial pressure
al concentration in dry gas phase
ntration in blood phase
e flow of blood
piratory exchange ratio (volume CO_2/volume O_2)

spired gas
red gas
gas
lar gas
e
r al
l en us
pillary

D Standard temperature and pressure dry (0 C 760 mm Hg)
S body temperature and pressure saturated with water vapor

al Symbols

Oxygen saturation of hemoglobin per cent
Suffix den ting total blood shunt in the lungs as defined in the text
HCO_3 The plasma bicarbonate content (mM/liter) of blood under standard condi
n s te at 37 C pH 7 4 and saturated with oxygen
S A body surface area m
p n n 0 113 W = 7 32 ft lb min

mbols used for the statistical treatment of data

Number of observations
ange Smallest and greatest observed value
Arithm ti mean
Standard devi ti n
Standard err r of the mean
Pr bab ity

II Introduction

The present investigation is ultimately concerned with the effects of G forces on the adaptation of cardiovascular and respiratory functions to exercise It represents a continuation of earlier experiments in this laboratory, in which both the tilt board and the human centrifuge have been used to expose the organism to changes in G vectors The most obvious and direct effect of exaggerated G stress is the increased hydrostatic pressure gradients in the circulation brought about by the increased effective weight of the blood Being the most mobile tissue in the body blood is easily displaced in the direction of the G force which may drastically change its normal distribution to the organs of the body

Practically all physiological research on the effects of exercise have so far been carried out in the normal gravitational environment, excepting certain experiments performed at 0 G in space vehicles However recent reports from this laboratory on the adaptability of the circulatory and respiratory systems to muscular exercise and increased G stress have demonstrated on the one hand the greater tolerance of the cardiovascular system to G stress in the head to seat direction brought about by leg exercise (Rosenhamer 1967) and on the other hand that the ability of healthy non athletes to perform exercise on the bicycle ergometer at work loads up to and including 900 kpm/ /min for 6 minutes was not seriously impaired by simultaneous exposure to 3 G (Bjurstedt and Rosenhamer 1967)

The present investigation has utilized the method of exaggerating the force of gravity to study the handicap caused by large hydrostatic pressures to cardiac and ventilatory performance during exercise Since the forces produced by accelerated motion are qualitatively indistinguishable from that of normal gravity the centrifuge is uniquely suited to making the influences of gravity more easily understood In order to elucidate certain G dependent characteristics of the physiological adaptation to work human subjects were investigated in the sitting position in the centrifuge cabin both in the resting condition and when performing leg exercise on a bicycle ergometer at different work loads To study the influence of increased gravitational stress on cardiovascular and ventilatory variables a force three times that exerted by normal gravity (3 G) was chosen

In the ensuing section (Chapter III) the background to the present in

vestigation is outlined and the problems defined Methodological details including the general design of the experiments procedures and techniques employed to approach the problems are described in Chapter IV The experimental results on the adjustments of cardiovascular and respiratory functions during exercise at the 3 G level, and comparisons with the corresponding results at normal gravity are given in Chapter V The results are discussed in Chapter VI Briefly the increments in cardiac output with the transition from rest to light and moderate exercise at the 3 G level were predominantly brought about by a prompt and marked increase in the stroke volume while heart rate was relatively unresponsive The utilization of the venous oxygen reserve was greater during increased gravitational stress The respiratory oxygen transport capacity at 3 G which was impaired due to alterations in pulmonary blood flow both throughout the lungs and regionally, showed only partial restoration with exercise the alveolar arterial oxygen pressure difference in effect becoming significantly greater than in the resting condition It is of interest to note that exaggeration of the force of gravity provoked certain alterations in cardiovascular and respiratory responses to exercise that have so far been inconspicious or difficult to demonstrate with changes of posture at normal gravity

In the following a brief survey is given of present concepts concerning the influence of gravitational vectors on the mode of adaptation of circulation and respiration to exercise This survey forms the background of the problems which are presented in the closing paragraphs of the two sections of this chapter

Effects of Gravitational Force upon Cardiovascular Adjustments to Exercise

Numerous investigations have shown that changes in posture can cause great quantities of blood to be interchanged between the intrathoracic compartment and the systemic capacitance vessels, particularly those of the lower extremities (for review see Gauer and Henry 1963) Furthermore it is generally agreed that the reduction of the stroke volume that accompanies a change of posture from supine to standing (for review see Wade and Bishop 1962) is caused by peripheral pooling of blood due to the influence of gravity Whereas there is good evidence that the size of the intrathoracic blood volume determines the functional reserves of the heart when the venous supply is momentarily curtailed (Gauer and Henry 1963), the possible relationship between changes in pulmonary blood volume and in cardiac output during exercise is a matter of debate (Marshall and Shepherd 1963)

The factors responsible for the rapid increase in cardiac output upon initiation of physical exercise have not been clearly defined Besides other control mechanisms for cardiac activity the Starling mechanism for augmented stroke volume through increased ventricular filling has repeatedly been emphasized Experiments in dogs (Guyton *et al* 1962) have indicated the importance for the increase in cardiac output during exercise of mechanical translocation of blood toward the heart by intermittent compression of the intramuscular vessels (*cf* Berne and Levy 1964) However experiments by Franklin Van Citters and Rushmer (1962) do not support the concept that changes in venous return represent a dominant mechanism for inducing changes in cardiac output and in a monograph by Rushmer (1961) it is suggested that under normal conditions blood flow may increase in all parts of the cardiovascular system more or less simultaneously without the need for

shifts in blood distribution Whether this concept was meant to apply also the circulatory adaptation to exercise in different body postures was not specifically stated Fishman (1963) suggested from considerable indirect evidence that the pulmonary blood volume increases during supine exercise *i.e.* from observations that the "central blood volume", as estimated by the Stewart Hamilton method increases (Braunwald and Kelly 1960) and that pulmonary compliance decreases (McIlroy, Marshall and Christie 1954) Further support for this view has been presented by Schreiner *et al* (1963) from studies on changes in the pulmonary blood volume, using the "double indicator method" (for review and application of this method, see Forsberg 1964) On the other hand Coucondilas and Shepherd (1963), in experiments on dogs, and Levinson Pacifico and Frank (1966) in experiments on human subjects, measured cardiopulmonary blood volumes by indicator dilution methods and concluded that no significant changes occurred with exercise It has been shown however that the rise in the diffusing capacity for carbon monoxide (D_{LCO}) during exercise is to an essential degree due to an increase of the volume of blood in the pulmonary capillaries (Johnson *et al* 1960, Johnson, Taylor and Lawson 1965) It has also been suggested that the maintenance of pulmonary capillary blood volume in the upright posture, like that of cardiac filling, is dependent upon the degree of filling of the central vascular compartment and that the leg muscle pump contributes to this filling (Daly, Krumholz and Ross 1965)

In a review on the volume and distribution of blood and their significance in regulating the circulation Sjöstrand (1953) emphasized the dependence of the adaptability of the circulation upon the blood volume contained in the heart and lungs According to the concept advanced the cardio pulmonary reserve blood seems to influence both the frequency and stroke volume of the heart *e.g.* during exercise and with changes in posture More recent investigations from Sjöstrand's group have dealt with the differences in the circulatory responses to exercise that can be observed in the supine as distinguished from the sitting position Thus Bevegård Holmgren and Jonsson (1960 1963) showed that both the stroke volume and cardiac output during leg exercise is considerably lower in the sitting than in the supine position while no significant difference in heart rate values was observed for oxygen uptakes above approximately 1 liter/min

In other studies the increase in stroke volume which occurs in normal subjects with leg exercise in the sitting position was found to be considerably smaller in patients with absence of venous valves (Bevegård and Lodin 1962), and likewise in patients with varicose leg veins (Grimby, Nilsson and Sanne 1964) In both studies an impaired efficiency of the leg muscle pump to

redistribute gravitationally shifted blood from the legs to the central circulation was suggested as an explanation of the obtained results

Whereas no significant differences in heart rate values were observed for oxygen uptakes above approximately 1 liter/min in normal subjects in the studies of Bevegård, Holmgren and Jonsson (1960 1963) this was the case in the afore mentioned studies on patients with abnormal veins In these patients the smaller stroke volumes during work in the sitting than in the supine position were partially compensated for by higher heart rates In the normal subjects the reduced stroke volume during exercise in the sitting position was compensated for by a higher arterio-venous oxygen difference resulting in unimpaired oxygen transport per pulse beat

That in sitting subjects a simulated increase of the force of gravity to three times its normal value (3 G) leads to a markedly reduced stroke volume has been demonstrated in centrifuge experiments by Wood s group at the Mayo Clinic (Wood *et al* 1961) who reported a decrease by approximately *37 per cent in the resting condition Light to moderate exercise at 3 G has* been shown by Rosenhamer (1967) and by Bjurstedt and Rosenhamer (1967) to be associated with higher heart rates than the corresponding work load at normal gravity At the transition from rest to work at 3 G the response of the heart rate was relatively sluggish as compared with the response at normal gravity It seemed likely that the higher absolute values in heart rate at 3 G exercise were compensatory for a deficient adaptation of stroke volume to exercise and that the relative unresponsiveness of the heart rate at the transition from 3 G rest to 3 G exercise reflected the initial but insufficient augmentation of stroke volume that occurred with the onset of exercise

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The afore mentioned studies at the 3 G level did not permit definite conclusions as to the more detailed mechanisms involved in the adaptation of cardiac and circulatory dynamics to exercise The present study was therefore undertaken to investigate the influence of increased gravitational stress upon the responses of cardiac output stroke volume and arterio-venous oxygen difference to light and moderate exercise with the force vector in the head seat direction magnified from 1 G to 3 G

G Dependence of Respiratory Adjustments to Exercise

Lilienthal *et al* (1946) and Filley Gregoire and Wright (1954) observed marked increases in the alveolar arterial (A—a) O difference during moderate

ly strenuous exercise Bartels *et al* (1955) and Asmussen and Nielsen (1960), however reported only small and inconsistent increments in this difference at similar levels of work Hesser and Matell (1965), on the other hand, observed slightly reduced A—a O_2 differences both during light and moderate exercise in the sitting position and pointed out that variations in results may be accounted for by postural influences as well as by differences in the techniques used for blood sampling and for correction of temperature effects

Studies by Lenfant 1964 indicate that as much as 85—90 per cent of the total A—a O_2 difference in the seated posture at normal gravity results from uneven distribution of ventilation perfusion in the lung (*cf* Hesser and Matell 1965) The dependence of the distribution of perfusion upon the gravitational factor has been clearly demonstrated for different body positions (West and Dollery 1960 Kaneko *et al* 1966 Although there is a qualitatively similar gravity-dependent gradient of ventilation (Ball *et al* 1962, Bryan, Milic Emili and Pengelly 1966 the perfusion gradient in the lung is markedly steeper Bryan *et al* 1964 It is accordingly generally believed that the dominant factor in creating the ventilation perfusion inequality and the resulting A—a O_2 difference under normal gravity conditions is the hydrostatically induced gradient of perfusion

West and Dollery 1960 and Bryan *et al* (1964) demonstrated that in the upright position a change from rest to exercise results in more uniform ventilation perfusion ratios This was shown to be due to a greater change in distribution of perfusion than of ventilation It should be pointed out however, that Read and Fowler 1964 by studying cardiogenic oscillations in the expired [illegible] in normal upright subjects during exercise, found a rather wide [illegible] individual changes in the redistribution of blood flow It was suggested that in resting normal subjects the small pulmonary vessels are in a variable state [illegible] motor tone and that this may influence the redistribution of blood flow with exercise in the upright body position Although the A—a O_2 difference was not measured in this study such variability in individual reactions may well [illegible] unt for variations in results with regard to exercise induced changes in this difference in the erect posture

It is well known that under resting conditions, inertial forces may produce gross shunting of blood in the dependent parts of the lungs leading to insufficient oxygenation of the arterialized blood (Barr Bjurstedt and Coleridge 1959 Barr 1962 Wood *et al* 1963) A fourfold increase in the [illegible] veolar arterial O_2 difference was reported by Barr (1963) at 5 G acting [illegible] ad to [illegible] direction Bjurstedt and Rosenhamer (1967) in experim[illegible] effec

of leg exercise in the sitting position at 3 G observed that the oxygen uptake per liter inspired air was lower than in exercise at normal gravity

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Whereas the nature of the disturbances in pulmonary function during increased gravitational stress is fairly well known for the resting condition there is little if any, information on the influence upon these disturbances of simultaneous muscular exertion with increased total blood flow through the lungs The present study therefore also aimed at analyzing the mode in which a change in the force vector from 1 G to 3 G in the apex base direction of the lungs modifies ventilation and pulmonary function during light and moderate exercise especially with regard to changes in ventilation perfusion relationships and in alveolar arterial O_2 differences

IV Methods

Introductory Remarks

Results obtained in previous studies (Rosenhamer 1967, Bjurstedt and Rosenhamer 1967) indicated that heart rate and respiratory variables attain relatively stable values within 3 to 4 minutes in the 3 G resting condition. Following the onset of moderate exercise (600 kpm/min), started one minute after attaining the 3 G level, there were no statistically significant differences in the values obtained for either V_E, V_O or R when measured during the 6th, 9th and 12th min of exercise. The heart rate, however, showed a progressive increase. The time course of the changes in the heart rate on the other hand indicated that cardiovascular compensatory reactions during 3 G exercise were largely completed within approximately 3 min in subjects with average physical work capacity at normal gravity. This conclusion was drawn from over 100 6 min work tests performed at 3 G in this laboratory for various work loads up to and including 900 kpm/min.

It was therefore conclud d that meaningful comparisons between the 1 G and 3 G values for the r piratory variables and heart rate would be possible if measured over sampling periods of 1—2 min following the 4th min of rest and 5th min [illegible] cise. In the case of blood gas levels and cardiac output, the temp [illegible] of these variables was studied over extended periods of tim [illegible]. For this purpose continuous recording techniques for [illegible] and S_O previously developed in this laboratory were adopted. For [illegible] techniques for continuous recording of arterial P_O and P_{CO} [illegible] for use in the spinning centrifuge. Likewise, a technique was [illegible] automatic repeated determinations at short intervals of cardiac output in subjects riding the centrifuge.

The fluctuations with time that were observed for S_O, pH, P_O and P_{CO} just prior to, during and immediately after the above mentioned sampling period were found to be sufficiently small to permit the time averaged values over these periods to be regarded as representative.

General Design of Experiments

The present study is based on two experimental series. They were both performed with the subjects seated in the cabin of a human centrifuge by means of which the effects of leg exercise on a bicycle ergometer could be investigated

Fig 1 Subject in position in the bicycle-ergometer assembly shown removed from the centrifuge In all experiments the assembly was accommodated in the centrifuge cabin with the *subject in the same position In the rotating centrifuge the cabin swings out freely so that* at constant speed the resultant G force remains in the same direction relative to the head *seat axis of the body as does the normal force of gravity with the centrifuge standing still.*
G levels used in the experiments 1 G (centrifuge stationary) and 3 G (centrifuge rotating at a speed that gives a resultant force of three times that of normal gravity as *measured in* the center of the cabin)

both at normal gravity and with the subjects exposed to a *simulated increase* of gravity to three times its normal value with the direction of the [illegible] (resultant) *force remaining unchanged relative to the body*[1] Fig 1 shows [illegible] position of the the subject which was the same at the two G levels (1 G and 3 G) The experimental conditions were similar in the two series in [illegible] logical responses to exercise at 300 and 600 kpm/min were [illegible] compared at the two G levels used However because of [illegible] predominantly technical nature cardiovascular adjustments and [illegible] adjustments were studied separately in the two series In [illegible] *rested supine for approximately* 60—90 min *before the* [illegible] begun G suits were not used

1st series The subject was positioned in the centrifuge [illegible] fuge started On attaining the 3 G level he *remained* [illegible]

Synonyms often used to indicate the magnitude and direct[illegible] "positive or "headward acceleration + 3 G Because of [illegible] the present experiments (see p 17) the magnitude of the [illegible] direction was in reality G × cos 13 (0 97 G) and 3 G [illegible] The small errors introduced by instead using 1 G and 3 G [illegible] neglected throughout the text

after which exercise at 300 kpm/min was commenced. Following exercise at this work load for 8—9 min the load was increased to 600 kpm/min lasting for 8—9 min. The centrifuge was then stopped, and the subject rested supine for 60—90 min. With the centrifuge stationary (1 G) the subject subsequently rested and exercised in the cabin at the same work loads and for the same time periods as at 3 G.

Determinations of cardiac output were made repeatedly in all experimental conditions by means of the indicator-dilution, single injection method. Four consecutive indicator-dilution curves were inscribed at 75 to 115 sec intervals in each of the experimental conditions, the intervals varying somewhat depending on the stability of the background dye concentration. In the resting conditions the first injection was given after 2.0 min and in the exercise conditions after 3.0 min. The curves obtained from these injections were regarded as wash-out curves and were discarded and the cardiac output was estimated from the mean values of the three following curves. In all exercise experiments *the four consecutive curves were obtained within* 5—6 min.

2nd series. [illegible] experimental conditions were the same as in the 1st series, except that the duration of each of the resting and two exercise experiments at 1 G and 3 G was 8 min. In this series the arterial pH and oxygen saturation as well as the arterial oxygen and carbon dioxide tensions were continuously [illegible] throughout the experiments.

[illegible] and heart rate were also recorded continuously in both [illegible] oxygen uptake and carbon dioxide elimination were [illegible] in both series over 2 min periods between the 5th and 7th min of [illegible] and 3 G rest and during the 6th or 6th and 7th min of the 1 G [illegible] and 3 G exercise experiments at each work load.

In both series the *change* in the magnitude of the force vector in the head-seat *axis produced upon rotating the centrifuge at the 3 G level was approximately* double that obtaining at normal gravity when an individual sits up from the lying-down position.

As reported elsewhere, the external work provided by the bicycle ergometer was essentially unaffected by the change in G level (Rosenhamer 1967). The characteristic work load discontinuity that appears during each 360° turn of the pedals in steady bicycling at normal gravity was somewhat altered at 3 G because of augmented fluctuations in the total potential energy of the legs. These repetitive load fluctuations, however, were small in relation to the total energy contained and dissipated in the ergometer and would have no significant influence on the average work load (Bjurstedt and Rosenhamer 1967).

TABLE I Individual anthropometric and functional data

Subj	Age years	Weight kg	Height cm	B S A[1] m²	Hb conc g/100 ml	PWC[2] kpm/min	Heart rate after 8 min standing beats/min
1	23	69	177	1 85	13 7	875	92
2	24	61	170	1 71	13 9	950	82
3	20	59	172	1 70	14 2	1050	99
4	22	67	183	1 87	12 6	975	88
5	25	62	180	1 79	13 5	775	105
6	27	74	181	1 94	12 4	1600	76
7	24	65	187	1 84	13 2	1125	79
[illegible]	25	70	189	1 90	14 6	850	95
[illegible]	25	63	176	1 77	14 3	950	104
10	27	[illegible]	190	2 07	14 2	1275	87
11	20	61	183	1 80	13 9	800	100
12	23	69	174	1 83	13 7	1200	94

From nomogram of Dubois and Dubois (1916)

PWC = physical working capacity at pulse rate 170 (Sjostrand 1947 Wahlund 1948) determined with the subject seated in the centrifuge cabin at 1G posture as in Fig 1

Subjects and Experimental Procedure

Twelve healthy male students served as test subjects of whom six (Nos 1 to 6) participated in both experimental series The series involving measurements of blood gas tensions was performed on ten subjects (Nos 1 to 10) and the series on cardiac output estimations on eight subjects (Nos 1 to 6 and 11 to 12) Individual dimensional and functional characteristics are presented in Table I

All experiments were performed in the morning after a light meal The 3 G experiments preceded the 1 G experiments by 60—90 minutes In three or more centrifuge runs the subjects had been made thoroughly familiar with the experimental conditions A Teflon catheter was first introduced percutaneously into the subject s right radial artery at the wrist (Barr 1961) for use with the recording of blood gas levels In those experiments which included cardiac output determinations an additional Teflon catheter (length 30 cm O D 1 7 mm I D 1 2 mm) was introduced percutaneously into a cubital vein and its tip placed in the axillary vein The subject then rested in the supine position for at least 20—30 minutes and was given 200 mg Heparin (Vitrum) intravenously to prevent clotting of the blood drawn through the sensing units during the ensuing experiments

With the subject seated in the centrifuge a modified aviator s oxygen mask was donned and tested until free from leakage The feet were attached to the pedals of a modified bicycle ergometer The axis of the bicycle pedals was at the level of the cabin seat the radius of the pedalling movement was 15 cm During all exercise experiments the pedal rpm was maintained at 60 The backrest of the seat was inclined backwards 13 from the vertical and the subject s occiput was in contact with a headrest His arms

and hands were supp rted in a c mfortable position with the el llowing exercise
The wrists were approximately at the level of the heart Recording to 600 kpm/min
six minutes before any given xp riment On starting the centrifug he subject rested
were permitted only during th t 20 sec The 3 G level was attain 1 G) the subject
and then remained constant u til the end of the experiment 17 e work loads and
subject was continuously remo t through a vent hole in the c
electric fan so that the temper tu was kept between 23 and
experiments n all experimental
injection method
at 75 to 115 sec

Techniques

vals varying some

Centrifuge All experiments were performed with the subjects p concentration In
of the human centrifuge at the Karolinska Institutet (radius ~ 0 min, and in the
and technical data see Cotzlinger and Helung 1955 Bjurs m these injections
experiments were made with the centrifuge standing still the 3 G and the cardiac
centrifuge rotating at 19 rpm

Ergometer An electrically braked bicycle ergometer (Holmgren following curves
was modified for use in the centrifuge cabin as described elsewhere obtained within
Bjurstedt and Rosenhamer 1967)

Recordings

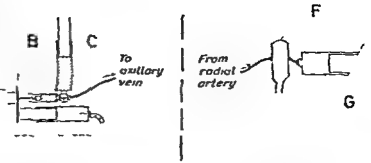
Cardiac output The indicator dilution technique was employed using indocyanine green Cardio Green® A standard dichromatic densitometer (Sutterer and Wood 196) was modified electrically to minimize drift and other disturbances The change in sensitivity to dye concentration at different dye background levels was checked once each experimental day during the calibration procedures In the calculation of al r u t ts no corrections were required for the variations observed in background dy c h tratio since they did not significantly affect the sensitivity of densitometer

A t chnique was developed for the repeated automatic assessment of dye-dilution c with the subject in the spinning centrifuge This technique employing remotely c tr ll d relays and a pneumatically activated dye injection syringe was designed t p rmit ardiac output determinations at short intervals in a reproducible fashion with t in onvenience to the subject A schematic diagram of the assembly used is h wn in Fig The injection syringe was calibrated gravimetrically to accommodate 1 0 ml to automatically re filling between each injection from a dye reservoir via a three way t pcock The whole system up the tip of the catheter in the axillary vein was fill d with dye and successive injections were made by displacement That the wash out d ly from the tip of the catheter varied negligibly between injections at intervals up t 1 0 sec was checked in model experiments using circulating plasma A 0.5 per cent a ueous solution of indocyanine dye was used The duration of each injection w 0 1 s conds and was constant within 0 01 seconds During the experiments the dye was always inject d near the end of an inspiration

Arterial bl d from the radial artery catheter (see Subjects and Experimen l Procedure p 17) was withdrawn through the densitometer cuvette at a co rf 20 ml m Between the recording of dye curves the blood sampled was re through the n catheter The dead space volume between the tip of the theter and th densitometer cuvette was 0 3 ml

TABLE I Individu[illegible]

Subj	Age years	W kg
1	23	69
2	24	61
3	20	59
4	22	67
5	25	62
6	27	74
7	24	65
8	25	70
9	25	63
10	27	[illegible]
11	20	[illegible]
12	23	69



...ram of as embly used for automatic dye dilution measurement of ...ive injections were made by dye displacement solenoids and motors ... from control room

... for interchangeable connection of injector syringe to venous ...oir

...tion of syringes for injection and refilling respectively of injector

E Plunger stops regulating volume of injectate

F Densitometer cuvette for continuous recording of dye concentration in flowing blood

G Constant flow infusion withdrawal syringe

For calibration undiluted arterial blood from the subject was used exclusively (a total of 75 ml for each calibration) The blood was collected in a volumetric flask and the automatic injection system was again used to introduce 1 07 ml of its remaining dye into the flask. Additional blood was then introduced to achieve three well-defined concentrations within the range encountered in the experiments Each experimental series at normal gravity and at 3 G was followed by calibration

The signals from the densitometer were fed to a photokymographic recorder to permit visual inspection of the continuous recordings The paper speed was 4 8 mm/sec the deflection for curves obtained at rest about 20—25 cm In addition these densitometer signals were fed into a 14 channel analog (FM) tape recorder (Ampex FR 100C) for storage of data (Fig 3) Subsequently off line digitalization of stored data was accomplished by use of an A/D converter (Hewlett Packard/Dymec) and tape punch (10 values per sec) For the final calculation of cardiac output an IBM 1401 computer was used (for details of methods and program in automatic computation of dilution curves see Wigertz Broman and Rosenhamer 1965 Broman and Wigertz 1966)

Other recordings All variables listed below were continuously monitored and inscribed on a photokymographic recorder (paper speed 1.25 mm/sec) throughout the experiments The arrangement used for the continuous recording of arterial pH O_2 saturation and O_2 and CO_2 tensions is schematically depicted in Fig 4 The rate of blood flow through the line of sensors was constant and amounted to 6 0 ml/min

Arterial O_2 saturation and pH were recorded by a cuvette oximeter (Wood Geraci and Croom 1948) and a glass reference electrode assembly previously developed by Barr and Bjurstedt (see Barr *et al* 1964) For the present experiments the pH assembl... was water jacketed and thermostated from a water bath regulated to 37 0 C 1/15-3...

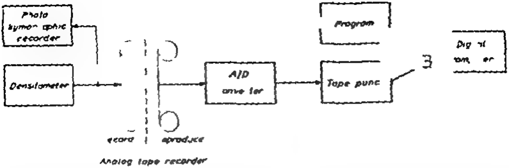


Fig 3 Schematic diagram of system used for on line recording and subsequent off line reproduction and processing of dye dilution data from centrifuge experiments (for further details see p 19) The photokymographic recorder was used in parallel for visualization and checking of the densitometer output signal

phosphate buffers for calibration of the pH electrode assembly were prepared according to Hastings and Sendroy (1924) and Van Slyke Weissiger and Van Slyke (1949) Repeated calibrations showed that the drift during any one of the experiments was smaller than 0 003 pH The cuvette oximeter was coupled to a logarithmic amplifier (Wiederhielm 1966 which gave a linear response following changes in the blood O_2 saturation F ... ration venous and arterial blood were sampled from the subject and drawn ... cally through the cuvette for subsequent analysis in duplicate by the Van ... thod The drift of the oximeter was virtually nil Following step input changes ... O_2 saturation and pH of the blood 90 per cent of full deflection was obtained in ... respectively

... oxygen and ... n dioxide tensions were recorded by means of electrodes according ... Clark (1956 and Severinghaus (1958) respectively The electrodes with a common 0 2 ml measuring chamber were arranged in series with the pH unit and ... inted in a water jacket with the water temperature regulated at 37 0 The combina... O_2 and CO_2 electrode assembly was designed according to Matell (1966) All values ... to arterial O_2 and CO_2 tension in this presentation have been corrected to ... temperature ... further Calculations p 21) For these corrections ... were used for measurement of rectal temperature as well as the temperature ... the recording system

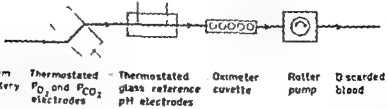


Fig 4 Pathway of continuously sampled arterial blood (sampling rate = 6 0 ml/min) for direct analysis of oxygen and carbon dioxide tensions pH and oxygen saturation by ... positioned in the centrifuge cabin

For cal the electrodes test gases of known oxygen and carbon dioxide fractional ions were b bled through distilled water for at least one hour The g s te water v er then in equilibrium with those in the gas as indicated y t tematic changes in readings with time The maximal drift as measured - the , ibration values obtained immediately before and after the experiments was $\pm$ 3 mm for P_O and $\pm$ 2 mm for P_{CO} The drift was not affected by the change from 1 G to 3 G The times required for 90 per cent deflection following step input changes in oxyg n and carbon dioxide tensions v ere 28 sec for the oxygen electrode and 18 sec for the carbon dioxide electrode

Inspired minute volume and tidal volume were continuously recorded by means of a dry rotary gas meter connected to an electronic pulse-counting device with analog output signals as described by Bjurstedt and Lonn (1960) The total resistance through the gas meter tubing and valves did not exceed 40 mm H_2O at the maximal flow rates encountered in the present experiments

Heart rate was obtained from electrodes attached to the chest and recorded by means of an instantaneous cardiotachometer (Sturm and Wood 1947)

Calculations

Cardiac output was computed according to the method of Kinsman Moore and Hamilton (1929) through the use of an automatic extrapolation and integration procedure Wigertz Broman and Rosenhamer 1965) During each cardiac output estimation (see General design of experiments 1st series) the mean heart rate level v as measured from the time of dye injection to the time of lowest concentration of the unextrapolated primary dye dilution curve

Expired minute volume was calcuted from the measured inspired volume with due corrections for R and the relative humidity of the inspired gas

O_2 uptake and CO_2 output were obtained by collecting the gas expired during predetermined periods successively in a series of bags A remotely-controlled solenoid actuated multiway stopcock allowed for the uninterrupted shunting of gas into a given bag during the desired period (Barr 1963) Expired gas samples were subsequently analyz d in duplicate for O_2 and CO_2 by Scholander s microtechnique (1947)

Arterio-venous oxygen difference was calculated as the quotient of the oxygen consumption and the cardiac output

pH levels measured over one minute intervals were corrected to rectal temperature using a factor of 0 0147 per degree C (Rosenthal 1948)

P_{O_2} and P_{CO} levels measured over one minute intervals were corrected to the actual body (rectal) temperature by the use of temperature coefficients according to Nunn *et al* (1965) Correction of the P_{O_2} for hemoglobin saturation v as included

$BHCO_{3\,s}$ (plasma bicarbonate of blood under standard conditions *i e* at 37 C pH 7 40 and saturated with oxygen) was estimated by first calculating the plasma bicarbonate content at 37 C from measured and temperature-corrected values of pH and P_{CO} by means of the Henderson Hasselbalch equation and then correcting this value to pH = 7 4 and S_O = 100 per cent according to Peters and Van Slyke (1931)

Effective alveolar O_2 tension and effective alveolar ventilation were obtained from the alveolar gas equation (Riley *et al* 1946) and the alveolar ventilation equation (Fenn Rahn and Otis 1946) substituting arterial for alveolar P_{CO_2}

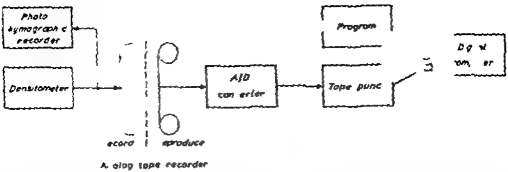


Fig 3 Schematic diagram of system used for on line recording and subsequent off line reproduction and processing of dye-dilution data from centrifuge experiments (for further details see p 19) The photokymographic recorder was used in parallel for visualization and checking of the densitometer output signal

phosphate buffers for calibration of the pH electrode assembly were prepared according to Hastings and Sendroy (1924) and Van Slyke Weissiger and Van Slyke (1949) Repeated calibrations showed that the drift during any one of the experiments was smaller than 0 003 pH The cuvette oximeter was coupled to a logarithmic amplifier (Wiederhielm 1956) which gave a linear response following changes in the blood O_2 saturation I ibration venous and arterial blood were sampled from the subject and drawn ically through the cuvette for subsequent analysis in duplicate by the Van method The drift of the oximeter was virtually nil Following step input changes O_2 saturation and pH of the blood 90 per cent of full deflection was obtained in sec respectively

tal oxygen and carbon dioxide tensions were recorded by means of electrodes accord Clark (1956 and Severinghaus (1958) respectively The electrodes with a common 0 ? ml measuring chamber were arranged in series with the pH unit and mounted in a water jacket with the water temperature regulated a 37 0° The combination) and CO_2 electrode assembly was designed according to Matell (1966) All values rep I for arterial O_2 and CO_2 tension in this presentation have been corrected to actual body temperature see further Calculations p 21) For these corrections thermistor units were used for measurement of rectal temperature as well as the temperature in the recording system

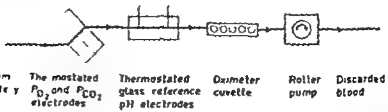


Fig 4 Pathway f c ntinu usly sampled arterial blood (sampling rate ~ 6 0 ml min) f r direct analys s f xygen an l carb n dioxi le tensions pH and oxygen saturation by sensing units positi ned n th centrifi ge cabin

Table II Comparison of circulatory data from experiments at normal gravity (1 G) and at 3 G Data were derived from individual averages obtained between the 5th and 9th min at each experimental condition (see foot notes) n = 8

			Rest[1]	Exercise	
				300 kpm/min	600 kpm/min
$\dot{V}_{O_2}$	1 G	M±SE	262± 9	937± 47	1521± 28
ml/min		Range	230—307	785—1233	1394—1659
STPD	3 G	M±SE	411± 23	1083± 40	1651± 48
		Range	317—500	913—1[illegible]12	1482—1872
	Diff	M±SE	149± 26	146± 55	130± 55
	3G—1G	P	< 001	< 05	< 05
Cardiac	1 G	M±SE	6 7± 5	1[illegible] [illegible]± 1 0	16 3± 1 0
output[4]		Range	5 3—8 5	9 3—17 4	11 4—20 5
liters/min	3 G	M±SE	5 1± 4	10 2± 5	14 1± 7
		Range	3 8—6 5	8 0—12 7	11 6—17 2
	Diff	M±SE	-1 6± 5	-[illegible] 0± 9	-2 2± 1 1
	3G—1G	P	< 05	< 05	< 05
Heart rate[4]	1 G	M±SE	71± 4	107± 6	136± 7
beats/min		Range	51— 89	71—1[illegible]3	92—155
	3 G	M±SE	115± 9	127± 9	159± 7
		Range	74—15[illegible]	88—162	124—181
	Diff	M±SE	44± 6	20± 5	23± 4
	3G—1G	P	< 001	< 01	< 001
Stroke	1 G	M±SE	96± 6	118± 12	124± 11
volume		Range	60—115	76—176	[illegible]6—171
ml	3 G	M±SE	47± 7	85± 11	92± [illegible]
		Range	25— 84	59—145	64—133
	Diff	M±SE	-49± 7	-33± 6	-32± 7
	3G—1G	P	< 001	< 001	< 01
a—$\bar{v}$ O	1 G	M±SE	4 1± 3	7 8± 5	9 6± 5
diff		Range	3 1—5 3	5 5—10 4	8 1—12 2
vol	3 G	M±SE	8 1± 5	10 5± 6	11 6± 4
		Range	6 2—9 8	8 5—13 4	9 7—13 0
	Diff	M±SE	4 0± 6	2 7± [illegible]	2 1± 6
	3G—1G	P	< 001	< 01	< 01

[1] Both the 1 G and 3 G resting conditions were preceeded by 60—90 min supine rest
6th to 7th min
5th to 9th min (mean of 3 consecutive determinations)
[4] Mean of heart rate levels during the three cardiac output determinations
Calculated from cardiac output and heart rate
Calculated according to the Fick principle

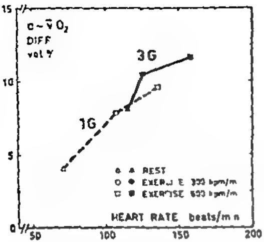


Fig. 9. Relationship of arterio-venous oxygen difference and heart rate during rest and exercise at 1 G and 3 G (based on group means from Table II, n = 8)

for exercise at 600 kpm/min. Fig. 9 depicts the interrelations between mean values for heart rate and $a-\bar{v}\ O_2$ difference at the two G levels. It can be seen that the $a-\bar{v}\ O_2$ difference increased with the heart rate at both G levels and that at 3 G the transition from rest to the mildest form of exercise yielded a better utilization of the venous oxygen reserve for a given increase in heart rate.

Respiratory Adjustments to 3 G Exercise

Total ventilation. Table III shows that the V_E values during 3 G rest and exercise were all higher ($P < 0.001$) than at normal gravity. The 3 G—1 G differences were larger during exercise (12.6 liters for 300 and 17.4 liters for 600 kpm/min) than at rest (6.6 liters/min), these values referring to V_E corrected for external dead space (Table III).

Tidal volume and respiratory rate. At rest, both variables displayed significantly higher mean values at 3 G than at normal gravity (Table III). Under exercise the tidal volume increased relatively less than the respiratory rate at 3 G as compared with 1 G. With exercise at 600 kpm/min there was no statistically significant difference in tidal volume between the two G levels.

Oxygen uptake. In both the resting and exercise experiments, the mean V_{O_2} was higher at 3 G than at normal gravity (Table III, *cf.* Table II), the differences being statistically significant. The 3 G exercise values for mean V_{O_2} were almost identical in the two experimental series. Furthermore, the rate of increase in mean V_{O_2} with a change from the slower to the higher load was approximately the same in both series and for both G levels. In the series accounted for in Table III, the mean increase from rest to 300 kpm/min at 3 G was 746 ml/min, 600 kpm/min resulting in a further increase of 574 ml/min.

TABLE III Comparison of respiratory data from experiments at normal gravity (1 G) and at 3 G Data were derived from individual averages over specified periods of sampling (see foot notes p 31) n = 10

			Rest[1]	Exercise[a]	
				300 kpm/min	600 kpm/min
$\dot{V}_E$ (measured) liters/min BTPS	1 G	M ± SE	10 1 ± 4	23 8 ± 8	36 5 ± 1 0
		Range	8 0—12 0	18 5—28 2	30 5—40 8
	3 G	M ± SE	16 8 ± 1 2	36 9 ± 1 5	54 7 ± 2 2
		Range	12 9—24 9	29 9—43 9	47 8—67 6
	Diff	M ± SE	6 7 ± 1 1	13 1 ± 1 9	18 2 ± 2 4
	3G—1G	P	< 001	< 001	< 001
$\dot{V}_E$ (corr)[a] liters/min BTPS	1 G	M ± SE	8 8 ± 4	22 3 ± [illegible]	34 9 ± 9
		Range	[illegible] 7—10 8	17 3—26 8	29 6—38 [illegible]
	3 G	M ± SE	15 4 ± 1 2	34 9 ± 1 4	52 3 ± 2 1
		Range	11 4—23 7	28 5—41 4	45 9—64 4
	Diff	M ± SE	6 6 ± 1.2	12 6 ± 1 8	17 4 ± 2 3
	3G—1G	P	< 001	< 001	< 001
V_T ml BTPS	1 G	M ± SE	627 ± 69	1247 ± 71	1794 ± 124
		Range	294—1044	971—1607	1374—2657
	3 G	M ± SE	926 ± 120	1406 ± 56	1818 ± 71
		Range	*587—1641*	*1160—1691*	*1527—2236*
	Diff	M ± SE	299 ± 121	159 ± 45	24 ± 83
	3G—1G	P	< 05	< 01	> 05
Breaths/ min	1 G	M ± SE	15 2 ± 1 3	18 2 ± 9	20 3 ± 1 5
		Range	8 0—22 7	13 3—22 5	11 1—26 5
	3 G	M ± SE	17 8 ± 1 4	25 2 ± 1 5	29 2 ± 1 8
		Range	12 5—26 2	16 9—33 8	23 1—40 1
	Diff	M ± SE	2 6 ± 1 0	7 0 ± 1 2	8 9 ± 1 7
	3G—1G	P	< 05	< 001	< 001
V_{O_2} ml/min STPD	1 G	M ± SE	282 ± 11	879 ± 36	1434 ± 41
		Range	230—353	706—1072	1180—1612
	3 G	M ± SE	336 ± 15	1082 ± 34	1656 ± [illegible]
		Range	260—390	960—1237	1477—1901
	Diff	M ± SE	54 ± 11	203 ± 46	222 ± [illegible]
	3G—1G	P	< 001	01	< 01
$\dot{V}_{CO_2}$ ml/min STPD	1 G	M ± SE	229 ± 7	734 ± 30	1209 ± [illegible]
		Range	188—274	573— 876	107[illegible]—1474
	3 G	M ± SE	277 ± 11	928 ± 36	1537 ± 51
		Range	203—321	787—1093	1257—1771
	Diff	M ± SE	48 ± 9	194 ± 47	[illegible]
	3G—1G	P	< 001	< 01	01
R (cont)	1 G	M ± SE	81 ± 01	84 ± 02	84 ± 01
		Range	77—87	77— 91	85—94

For foot notes see p 31

Table III Cont

			Rest[1]	Exercise[2]	
				300 kpm/min	600 kpm/min
	3 C	M ± SE	83 ± 02	86 ± 01	93 ± 0?
		Range	77—94	81—89	88—1 02
	Diff	M ± SE	0? ± 02	02 ± 02	04 ± 02
	3C—1C	P	> 05	> 05	> 05
S_{aO_2} %	1 C	M ± SE	96 1 ± 1	95 8 ± 1	95 5 ± 2
		Range	95 4—96 5	95 3—96 4	94 2—96 0
	3 C	M ± SE	96 0 ± 3	94 1 ± 3	92 2 ± 6
		Range	94 9—97 2	92 8—95 5	88 4—95 0
	Diff	M ± SE	−1 ± 3	−1 7 ± 3	−3 3 ± 6
	3C—1C	P	> 05	< 001	< 001
P_{aO_2} mm Hg	1 C	M ± SE	88 5 ± 9	87 2 ± 1 0	86 2 ± 1 2
		Range	84 7—93 6	83 4—91 9	78 7—91 7
	3 C	M ± SE	84 8 ± 2 2	76 1 ± 1 7	71 6 ± 2 1
		Range	74 1—94 1	67 6—86 3	59 6—84 2
	Diff	M ± SE	−3 7 ± 2 3	−11 1 ± 1 5	−14 6 ± 2 4
	3C—1C	P	> 05	< 001	< 001
Art pH	1 C	M ± SE	7 403 ± 005	7 386 ± 005	7 372 ± 007
		Range	7 382—7 493	7 369—7 415	7 349—7 403
	3 C	M ± SE	7 444 ± 013	7 387 ± 006	7 362 ± 007
		Range	7 379—7 500	7 363—7 415	7 335—7 413
	Diff	M ± SE	041 ± 010	001 ± 003	−010 ± 005
	3C—1C	P	< 01	> 05	~ 05
P_{aCO_2} mm Hg	1 C	M ± SE	40 1 ± 8	43 2 ± 8	44 1 ± 9
		Range	35 5—43 8	39 0—46 6	39 8—47 7
	3 C	M ± SE	35 3 ± 1 4	41 9 ± 1 1	42 0 ± 1 3
		Range	29 6—41 9	36 1—45 7	34 3—46 3
	Diff	M ± SE	−4 8 ± 1 1	1 3 ± 5	2 1 ± 7
	3C—1C	P	01	05	< 05
$BHCO_{3_{aa}}$ mM/liter	1 C	M ± SE	25 3 ± 5	25 5 ± 5	24 6 ± 5
		Range	22 7—27 9	23 0—27 8	21 6—27 4
	3 C	M ± SE	26 0 ± 1 0	24 4 ± 8	22 3 ± 8
		Range	19 2—30 8	21 3—28 1	17 6—26 0
	Diff	M ± SE	7 ± 8	1 1 ± 2	−2 3 ± 4
	3C—1C	P	05	001	< 001
Eff $\dot{V}_A$[4] liters/min BTPS	1 C	M ± SE	4 9 ± ?	14 6 ± 5	24 9 ± 6
		Range	4 2—5 7	12 7—17 1	22 0—27 4
	3 C	M ± SE	6 9 ± 4	19 2 ± 9	31 8 ± 1 3
		Range	4 2—9 3	16 1—24 9	27 2—40 3
	Diff	M ± SE	2 0 ± 4	4 6 ± 1 0	6 9 ± 1 4
	3C—1C	P	001	01	001

Table III Cont.

			Rest[1]	Exercise[2]	
				300 kpm/min	600 kpm/min
Eff $\frac{V_A}{V_E} \times 100$	1 G	M ± SE	[illegible] ± 3	66 ± 2	[illegible] ± 2
		Range	44—68	60—73	58—8[illegible]
	3 G	M ± SE	45 ± 2	55 ± 2	61 ± 2
		Range	3[illegible]—[illegible]	44—63	50—[illegible]8
	Diff	M ± SE	-11 ± 3	-11 ± 2	-11 ± 1
	3G—1G	P	< 01	< 001	< 001
V_{Dphys} ml BTPS	1 G	M ± SE	2[illegible]1 ± 2	4[illegible]5 ± 20	495 ± 30
		Range	95—303	200—568	274—612
	3 G	M ± SE	505 ± 76	625 ± 25	[illegible]02 ± 31
		Range	309—995	462—716	560—889
	Diff	M ± SE	234 ± [illegible]3	200 ± 24	206 ± 24
	3G—1G	P	< 01	< 001	< 001
$\frac{V_{Dphys}}{V_T} \times 100$	1 G	M ± SE	38 ± 2	32 ± 1	2[illegible] ± 2
		Range	26—50	25—37	17—40
	3 G	M ± SE	49 ± 2	4[illegible] ± 2	[illegible]9 ± [illegible]
		Range	40—58	35—53	31—48
	Diff	M ± SE	11 ± 2	10 ± 2	11 ± [illegible]
	3G—1G	P	< 01	< 001	< 001
Eff P_{AO_2} mm Hg	1 G	M ± SE	102 6 ± 9	100 1 ± 1 0	101 5 ± 1 1
		Range	95 6—106 2	9[illegible] 9—101 9	94 4—10[illegible] 5
	3 G	M ± SE	108 8 ± 1 9	102 7 ± 1 1	105 8 ± 1 [illegible]
		Range	98 7—116 2	99 1—109 0	99 6—114 8
	Diff	M ± SE	6 2 ± 1 8	2 [illegible] ± 1 4	4 3 ± 1 1
	3G—1G	P	< 01	< 05	01
Eff P_{AO_2}—P_{aO_2} mm Hg	1 G	M ± SE	14 1 ± 1 3	1[illegible] 9 ± 1 1	15 3 ± 1 1
		Range	8 1—19 8	[illegible] 8—17 9	9 5—20 7
	3 G	M ± SE	24 0 ± 2 [illegible]	[illegible] 6 ± 2 1	34 [illegible] ± [illegible] 1
		Range	15 0—37 3	13 3—35 [illegible]	22 9—4[illegible] 8
	Diff	M ± SE	9 9 ± 1 9	13 7 ± 1 9	18 9 ± 2 0
	3G—1G	P	< 001	< 001	001

Data refer to the last two min of the 1 G and 3 G resting periods both preceded by 60—90 min supine rest

Data refer to the 6th min of the 1 G and 3 G exercise periods

Corrected for external dead space = 80 ml

Plasma bicarbonate content of blood under standard condition i e at 37 C pH 7 40 and saturated with oxygen

Calculated as (V_{CO} × 863)/P_{CO}

Computed from the Bohr formula substituting arterial for alveolar CO

Calculated as $P_{IO} - P_{CO} \left(F_{IO} + \frac{1-F_{IO}}{R}\right)$

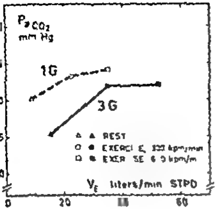


Fig. III. Arterial carbon dioxide tension (P_{aCO_2}) plotted against respiratory minute volume ($\dot{V}_E$) during rest and exercise at 1 G and 3 G (based on group means from Table III, n = 8).

Carbon dioxide elimination and respiratory exchange ratio. The mean $\dot{V}_{CO_2}$ increased with the work load at essentially the same rate as $\dot{V}_{O_2}$, so that the mean R values did not differ significantly between 1 G and 3 G either at rest or at the two work loads (Table III).

Arterial oxygen saturation and P_O. During exercise, both variables were found to be significantly lower at 3 G than at normal G (Table III). The individual P_{aO_2} values at the highest work load at 3 G varied between 60 and 84 mm Hg

TABLE IV. Total venous admixture and arterial oxygen deficit during the 6th min of the

			Rest	Exercise	
				300 kpm/min	600 kpm/min
Venous admixture[a]	1 G	M ± SD	6 4 ± 1 9	4 0 ± 1 4	3 5 ± 8
		P			
% cardiac output	3 G	M ± SD	5 0 ± 2 5	5 7 ± 1 9	8 0 ± 2 3
		P			
(n = 8)	Diff 3G − 1G	M ± SD	1 4 ± 2 8	1 7 ± 1 8	4 5 ± 2 4
		P	05	< 05	01
Arterial oxygen deficit[b]	1 G	M ± SD	27 ± 08	28 ± 7	34 ± 09
		P			
vol % O_2	3 G	M ± SD	43 ± 12	67 ± 19	1 07 ± 35
		P			
(n = 10)	Diff 3G − 1G	M ± SD	16 ± 13	39 ± 18	73 ± 36
		P	01	001	001

[a] Calculated as $100 \dfrac{C_{c'O_2} - C_{aO_2}}{(C_{c'O_2} - C_{aO_2}) + a\text{-}\bar{v}\,O_2 \text{ diff}}$

[b] Estimated as $(C_{c'O_2} - C_{aO_2})$

while at normal gravity the range was 79 to 92 mm Hg For the resting condition on the other hand there was no significant difference between the 1 G and 3 G values for either variable

Arterial P_{CO} Lower mean values were obtained at 3 G than at normal gravity (Table III), but during exercise they differed by only 1 3 and 2 1 mm Hg at the two work loads respectively At 3 G the mean values were found to change very little with the transition from 300 to 600 kpm/min (group mean difference 0 1 mm Hg) while a marked increase was observed following the transition from rest to 300 kpm/min (mean difference 6 6 mm Hg $P < 0\,001$) The interrelationships of P_{aco} and ventilation at 1 G and 3 G are depicted in Fig 10, which shows that there was a true hyperventilation in all the 3 G conditions especially marked in the 3 G resting condition (*cf* below "Effective alveolar ventilation")

Arterial pH and standard bicarbonate As was the case for the arterial P_{CO} the hydrogen ion concentration was significantly lower at 3 G rest than at 1 G rest (Table III) With 3 G exercise there was an acidotic shift in all subjects This was also the case at normal gravity although less pronounced, so that the group mean pH values at 300 kpm/min were the same at the two G levels and the 600 kpm/min values differed by only 0 01 pH units Table III shows that

1 G and 3 G resting and exercise conditions

Diff 300 kpm//min—rest	Diff 600 kpm//min—rest	Diff 600 kpm//min—300 kpm/min
-? 4± 7	-2 9±1 3	- 5± 7
< 001	< 001	> 05
7±1 5	3 0±2 2	2 3± 8
> 05	< 05	< 001
01± 04	07± 04	06± 05
> 05	< 01	< 01
24± 16	64± 31	40± 18
< 01	< 01	< 001

the mean $BHCO_{3s}$, during the highest work load was significantly lowered by 2 3 mM liter by the change from 1 G to 3 G

The *effective alveolar P_O* increased by an average of 6 mm Hg ($P < 0.01$) to 109 mm Hg with the change from 1 G rest to 3 G rest The exercise values at both normal gravity and 3 G were slightly reduced when compared with the resting values at either G level somewhat more with 300 than with 600 kpm/min The 3 G exercise values however, were significantly elevated over the corresponding 1 G values Table III)

The *effective alveolar ventilation* was significantly higher at 3 G than at 1 G during both rest and exercise Table III) At 3 G it increased by 12 3 liters/min with the transition from rest to 300 kpm min and by another 12 6 liters/min at the highest work load Corresponding increases at 1 G were 9 7 and 10 3 liters min respectively These increments were significant ($P < 0.001$) at both G levels The Eff V_A to V_E ratio increased with exercise at both G levels It was lower at 3 G than at 1 G ($P < 0.01$), however, throughout both rest and exercise indicating an impaired efficiency of pulmonary function at 3 G

The *physiological dead space* was larger at the higher G level, both during exercise and at rest Table III At 3 G the resting value increased by 86 per cent over that obtained at normal gravity With exercise at 3 G, $V_{D_{phys}}$ increased by 24 and 39 per cent at the two work loads respectively The fact that the total ventilation increased more than the effective alveolar ventilation with a change from 1 G to 3 G both in the resting and exercise conditions is reflected in the V_D to V_T ratios which were higher at 3 G (Table III)

S... ... ares for blood gas levels during 1 G and 3 G exercise As presented above and in Table III the values obtained for the respiratory variables refer to the 5th and 6th min of observations at rest and to the 6th min of each work load To estimate the degree of temporal stability that the exercise values for $S_{a_{O_2}}$, $P_{a_{O_2}}$, $P_{a_{CO_2}}$ and pH represented a t test was applied to the intraindividual differences obtained between the 5th and 6th min for these variables at both G levels No significant difference was present for any of the aforementioned variables except for $S_{a_{O_2}}$ in one instance (1 G exercise at 300 kpm min where the 6th min mean value was 1 0 per cent higher than that for the 5th min with P 0 05

The *... ... admixture* in per cent of the cardiac output did not differ significantly between 1 G and 3 G when calculated for rest and for 300 kpm min Table IV For 600 kpm min however the 3 G value was significantly elevated by 4 5 per cent over the corresponding 1 G value ($P < 0.01$ It represented an average increase over the 3 G resting value by 3 0 per cent which was likewise significant P 0 05 By contrast the 1 G values for

300 and 600 kpm/min were significantly smaller than the 1 G resting value by 2.4 and 2.9 per cent, respectively.

The *arterial oxygen deficit* showed increments with exercise at both G levels; they were markedly pronounced at 3 G. This was the case both when the oxygen deficit was expressed in vol % (Table IV) or in ml/min (as depicted in Fig. 13).

VI Discussion

In view of the fact that the G force components directed along each thigh and leg were small both at 1 G and at 3 G it was thought justified for the following discussion to consider the over all circulatory effects of a change from one G level to the other to be dependent mainly on the magnitude of the G force along the head seat axis of the body

Work Performed by the Leg Muscles at 3 G

Theoretically a change in the G level may influence the demand of oxygen of the working leg muscles both through alterations in the external work load and through changes in the effective weight of the leg tissues

As mentioned in "General Design of Experiments" (p 16), the ergometer system was unaffected by the change in G force, *i e* the external work load afforded by the ergometer was the same at 1 G and 3 G The work load discontinuity that characterizes bicycling during each 360 turn of the pedals due to fluctuations in the potential energy of the legs increases with the magnitude of the G force However as analyzed by Bjurstedt and Rosenhamer (1967) the magnitude of these work load fluctuations is not likely to significantly influence the average metabolic need of the working muscles There remains the possibility that G induced changes in the "internal environment" of the working muscles through inertial displacement of blood and tissue, might affect their mechanical efficiency and thereby the total oxygen consumption Bjurstedt and Rosenhamer felt that this factor was practically of no importance at least not in the body position employed *i e* with the lower limbs principally oriented transversely to the acting force Consequently their observation that the total oxygen consumption for a given external work load was higher at 3 G than at normal gravity (*cf* also the present results p 28) was attributed mainly to greater metabolic demands of postural and respiratory muscles

Influence of Various Experimental Conditions in the 3 G Experiments

In a previous study Bjurstedt and Rosenhamer 1967) exercise at 1 G and 3 G at the same work loads and of similar duration as in the present experiments yielded no systematic differences in values for various respiratory vari

ables heart rate levels, or blood lactate concentrations, when the sequence of the 1 G and 3 G experiments was reversed However in the present investigation the experimental series on respiratory adjustments was associated with a continuous blood loss, which although small might theoretically be expected to have influenced the results obtained at 3 G as the hydrostatically induced displacement of blood and a reduction of total blood volume would exert additive effects on G tolerance (*cf* Gauer and Thron 1962) In fact, when in a pilot study the 1 G experiments preceded those at 3 G, there were several incidents of visual impairment or impending loss of consciousness after about 3—4 minutes of rest at high G It was also observed that during exercise under these conditions the test subjects regularly experienced a more marked feeling of general fatigue Therefore to avoid even small hemorrhages prior to the present 3 G experiments they were always performed before the 1 G experiments

To analyse whether the small blood loss that could not be avoided in one of the two series of experiments at 3 G accounted for in Methods (pp 15—16) had any significant influence on the results obtained the t test was applied to the intra individual differences observed between the two 3 G series This analysis revealed no significance probabilities below the 0 05 level for either respiratory minute volume, tidal volume respiratory rate oxygen uptake or carbon dioxide elimination indicating that there were no adverse effects on the afore mentioned variables of the blood loss at the 3 G level However, the heart rate was found to be significantly higher in the experiments with blood loss This was the case both during rest and at the two levels of exercise (mean differences 18 16, and 14 beats/min respectively, $P < 0\ 01$) The experiments at normal gravity showed no such difference Since the 3 G experiments were always performed first the total blood loss at the completion of these experiments never exceeded 130 ml Bleeding of about 10 per cent of the total blood volume has been shown by Gullbring *et al* (1960) to cause within one hour a decrease in the physical working capacity (PWC_{170} as estimated according to Sjostrand 1947) of 8 5 per cent in the sitting position at normal gravity Although in the present investigation the blood loss during the course of the 3 G experiments was much smaller the higher heart rates during 3 G exercise with blood loss (second series) indicate an effect at this G level similar to that demonstrated by Gullbring *et al*

Exercise at normal gravity has been shown to be associated with a reduction of the plasma volume (Holmgren 1956 Uehlinger and Buhlmann 1961 Konig and Zollner 1961) Uehlinger and Buhlmann suggested additive effects of exercise and orthostasis on transcapillary fluid losses from observations of a greater reduction of the plasma volume during exercise in the sitting posi

of the differences between successive determinations, expressed in terms of standard deviation, was found to be 6 7 and 9 6 per cent of the mean cardiac outputs at 1 G and 3 G, respectively, during both rest and exercise Corresponding values for the stroke volume were 7 3 and 10 2

The variation between repeated cardiac output estimates was of the same order of magnitude as obtained by Sleeper *et al* (1962) and Hanson and Tabakin (1964) for rest at normal gravity They were smaller, however, when compared with corresponding observations during exercise by Hanson and Tabakin (1964), who reported variations ranging between 10 and 30 per cent of the mean cardiac output In the latter investigation 7—11 per cent of the variation was reported to be inherent in the method and related to curve measurement error The electronic data processing method used for estimation of the cardiac output in the present study has presumably played a role in reducing such error

Cardiac output and stroke volume at 1 G rest and 3 G rest In the resting condition at 3 G the stroke volume was reduced to 49 per cent Thus curtailment of stroke volume was somewhat greater than that observed by Wood *et al* (1961), who reported a decrease to 63 per cent at the same G level Although in the present experiments the heart rate was markedly accelerated at 3 G (see below) the increase was not sufficient to maintain the cardiac output which fell to 76 per cent of the value obtained at normal gravity This reduction in cardiac output agrees well with that observed by Wood *et al*, who reported a decrease to 82 per cent of the value obtained at normal gravity

The observed reductions in the stroke volume and cardiac output in the resting condition with a change from 1 G to 3 G can be attributed to defective ventricular filling secondary to considerable, hydrostatically induced displacement of blood volume into dependent portions of the body (*cf* below, p 43) In comparison with resting at normal gravity resting at 3 G may therefore be interpreted as being associated with gross functional hemorrhage into the dependent vascular bed

Adjustments of cardiac output and stroke volume to exercise at 1 G and 3 G When performing work at increasing loads on the bicycle ergometer, while remaining at the 1 G level stroke volume increased only moderately Thus at 300 kpm/min it rose to 123 per cent and at 600 kpm/min to 129 per cent of the resting value Table II Fig 8 shows that the demand on the circulation during exercise at normal gravity was met by a more marked increase in heart rate than in stroke volume

When starting the same exercise pattern at 3 G, the stroke volume, which was initially much smaller than at normal gravity showed a striking increase its value at 300 kpm min amounting to no less than 181 per cent of the 3 G resting value Thereafter the stroke volume increased relatively little, so that

the value for 600 kpm/min was 191 per cent of the 3 G resting value (Table II) Fig II shows that, at the 3 G level cardiac adjustment to low intensity work was brought about by a much more marked increment in stroke volume than in heart rate, so that the mode of adaptation of these variables to low-intensity work greatly differed at the two G levels However Fig II also shows that with the further increase in work load the increments in stroke volume in relation to heart rate were about the same at 3 G as they were at normal gravity It can be seen that the reduction of the stroke volume at 3 G was about the same at the two work loads (33 and 32 ml at 300 and 600 kpm/ /min respectively *cf* Table II) As a consequence cardiac output at 3 G exercise was reduced by 2 0 and 2 2 liters/min respectively

The response patterns of stroke volume heart rate and cardiac output with a change from rest to leg exercise at the 3 G level can best be explained by the assumption that even with low intensity work a large volume of blood pooled in dependent regions is redistributed into the intrathoracic spaces by the action of the leg muscle pump

Supporting evidence for the role of the leg muscle pump in the maintenance of adequate cardiac output and stroke volume during exposure to 3 G has been presented by Rosenhamer (1967) who demonstrated the importance of leg exercise for the prevention of visual impairment impending loss of consciousness and other signs of failing circulation at the 3 G level and by Bjurstedt and Rosenhamer (1967) who reported that a change from rest to low intensity work at 3 G was associated with a much weaker heart rate response than at normal gravity (increase by 12 per cent at 3 G by 40 per cent at 1 G) indicating a relatively greater change in stroke volume The results from the last mentioned and present investigations demonstrate that an unusually high heart rate is required to achieve an adequate cardiac output at a given work load if compared with the corresponding heart rate responses at normal gravity

Fig 7 shows that for any given oxygen uptake the cardiac output was about 3 liters/min lower at the higher G level This effect is analogous to although more marked than that observed by Bevegård Holmgren and Jonsson (1960), Reeves *et al* (1961) and McGregor Adam and Sekelj (1961) with change from the supine to the upright position at normal gravity

The observation on the behavior of the stroke volume at the transition from rest to the mildest form of exercise at 3 G is of special interest when compared with the results of certain earlier investigations at normal gravity Thus cardiac responses to exercise have been studied as modified by changing the body position from supine to upright which produces an increase in the vector of gravity in the head seat direction from 0 G to 1 G (for reviews

see Wade and Bishop 1962, Gauer and Thron 1965) Bevegard Holmgren and Jonsson (1960) found a 41 per cent increment of the stroke volume on transition from rest to bicycle exercise (250 to 450 kpm/min) in the sitting position (legs oriented mainly downwards) whereas Wang, Marshall and Shepherd (1960) observed a 69 per cent increase with moderate exercise on the treadmill In both these studies considerably smaller changes were noted when exercise was started in the recumbent position The present study demonstrates that the response of the stroke volume to light exercise becomes clearly more marked (increase = 81 per cent when the magnitude of the force vector in the head seat direction is increased to 3 G

The present results emphasize the importance of the central blood pool and the filling of the heart for the response patterns of cardiac dynamics to the circulatory stress of exercise Thus the absolute values of the stroke volume were smaller during both rest and exercise in the present 3 G experiments than have been reported as typical for any posture at normal gravity (*cf* Wade and Bishop 1962) It is interesting to note that this was the case despite the fact that the vessels of the legs were oriented mainly transversely to the effective G force The explanation for the smaller stroke volumes in the present 3 G experiments seems to be, that any tendency toward improved ventricular filling due to the lack of an effective hydrostatic gradient along the leg vessels, was outweighed by downward drain of blood caused by the exaggerated hydrostatic pressure gradient along the head seat distance

G dependence of heart rate during leg exercise Asmussen Christensen and Nielsen were able to establish experimentally already in 1939 that the heart rate assumes higher values in the erect posture than in the recumbent both at rest and during arm exercise More recent investigations (for review see Gauer and Thron 1962) have not shown such a clear postural difference in heart rate with leg exercise where the hydrostatically induced reduction of stroke volume at rest is more likely to be at least partially corrected by exercise through the action of the muscle pump This can be exemplified by the findings of Bevegard Holmgren and Jonsson (1960) and Bevegard Freyschuss and Strandell 1966) who observed that there was no significant difference between the heart rate reached in sitting and supine leg exercise Nor did Hellstrom and Holmgren (1966) find any significant effect of posture on the heart rate per work load in 49 male subjects although an almost significant difference was obtained in 12 female subjects That a clearcut difference was observed in the present experiments indicates that the heart rate response to leg exercise is in fact G-dependent, but that the unmasking of such a difference may require an increase in the effective G force vector beyond the 1 G level

In summing up the following conclusions can be drawn from the present

results on the mode of adaptation of cardiac dynamics to exercise at the 1 G and 3 G levels In this study as in investigations of the effects of changes in posture, the action of G forces constitutes the physiologically significant factor In both cases alterations occur in the magnitude of the force vector in the head seat or head foot direction of the body the immediate effect of which is to change the magnitude of hydrostatic pressure differences in long uninterrupted columns of blood The results obtained have clearly demonstrated that a simulated increase of gravity to three times its normal value acting in the head seat direction on sitting individuals, not only markedly lowers the stroke volume and the cardiac output at rest but also prevents their exercise values from reaching those obtained at normal gravity

That larger exercise values for stroke volume and cardiac output and lower values for heart rate have usually been reported for the upright or sitting position than were observed in the present 3 G experiments can best be explained by the assumption that the smaller effective G vector in the head seat direction limited the drain of blood away from the intrathoracic spaces A simple calculation shows that the maximum change of the force vector on altering the body position from supine to erect (0 G→1 G) amounts to only half of that attained in the present experiments (1 G→3 G)

Redistribution of Blood Volume

The marked changes observed in stroke volume with transition from rest to low intensity work at both the 1 G and 3 G levels focusses attention on the concomitant changes in the blood volume available for ventricular filling It should be made clear at the outset that the validity of the indicator dilution method as used for determination of central blood volume (CBV) according to Hamilton *et al* (1932) has been debated especially with regard to the effects of posture and exercise Certain aspects of the technique used in determinations of CBV have been investigated notably by Marshall and Shepherd, who in a paper published in 1961 wrote that when either the injection site or the sampling site is peripheral any maneuver that causes redistribution of systemic blood flow such as exercise change of posture or general anesthesia *will be associated with a change in the calculated CBV Attempts to equate* this change quantitatively or even qualitatively with a change in the volume of blood in the lung vessels are however fraught with hazard

Nevertheless it seemed of interest to utilize the dye dilution curves used for determinations of cardiac output also for a comparison between CBV in the 1 G and 3 G experiments applying the method described by Hamilton *et al* It was found that in the resting condition the calculated CBV decreased by an

see Wade and Bishop 1962, Gauer and Thron 1965) Bevegard, Holmgren and Jonsson (1960) found a 41 per cent increment of the stroke volume on transition from rest to bicycle exercise (250 to 450 kpm/min) in the sitting position (legs oriented mainly downwards) whereas Wang, Marshall and Shepherd (1960) observed a 69 per cent increase with moderate exercise on the treadmill In both these studies considerably smaller changes were noted when exercise was started in the recumbent position The present study demonstrates that the response of the stroke volume to light exercise becomes clearly more marked (increase = 81 per cent) when the magnitude of the force vector in the head seat direction is increased to 3 G

The present results emphasize the importance of the central blood pool and the filling of the heart for the response patterns of cardiac dynamics to the circulatory stress of exercise Thus the absolute values of the stroke volume were smaller during both rest and exercise in the present 3 G experiments than have been reported as typical for any posture at normal gravity (*cf* Wade and Bishop 1962) It is interesting to note that this was the case despite the fact that the vessels of the legs were oriented mainly transversely to the effective G force The explanation for the smaller stroke volumes in the present 3 G experiments seems to be that any tendency toward improved ventricular filling due to the lack of an effective hydrostatic gradient along the leg vessels was outweighed by downward drain of blood caused by the exaggerated hydrostatic pressure gradient along the head seat distance

G dependence of heart rate during leg exercise Asmussen, Christensen and Nielsen were able to establish experimentally already in 1939 that the heart rate assumes higher values in the erect posture than in the recumbent both at rest and during arm exercise More recent investigations (for review see Gauer and Thron 1962 have not shown such a clear postural difference in heart rate with leg exercise where the hydrostatically induced reduction of stroke volume at rest is more likely to be at least partially corrected by exercise through the action of the muscle pump This can be exemplified by the findings of Bevegard Holmgren and Jonsson (1960) and Bevegard, Freyschuss and Strandell (1966), who observed that there was no significant difference between the heart rate reached in sitting and supine leg exercise Nor did Hellstrom and Holmgren (1966) find any significant effect of posture on the heart rate per work load in 49 male subjects although an almost significant difference was obtained in 12 female subjects That a clearcut difference was observed in the present experiments indicates that the heart rate response to leg exercise is in fact G dependent but that the unmasking of such a difference may require an increase in the effective G force vector beyond the 1 G level

In summing up the following conclusions can be drawn from the present

results on the mode of ada un have been larger, but presumably not smaller nd 3 G level d

From the above considerations it can be concluded therefore that the considerable reduction in the calculated resting CBV obtained with the change om 1 G to 3 G is likely to have been representative of the actual blood volume at was shifted into the lower portion of the body due to the increased hydrostatic pressure gradient along the head seat axis and that any changes t the blood flow in the sampling arm would tend to lead to an under timation of caudad displacement of blood volume It is likewise concluded at the redistribution of blood volume back into the intrathoracic spaces uring exercise as judged from the calculated changes in CBV at the two G evels reflected the much greater percentual increase in stroke volume that occurred already with low intensity work at 3 G as compared with the corresponding increase at normal gravity (*cf* Fig 8)

Oxygen Transport

The reduction of the resting stroke volume to about half its value with the change from normal gravity to 3 G was compensated for by a doubling of the a—$\bar{v}$ O_2 difference (Table II) so that the heart rate at 3 G rest was increased in direct proportion to the oxygen uptake the oxygen pulse *i.e.* the oxygen uptake per pulse beat remaining essentially unchanged Fig 9 shows that the increased demand on the oxygen transport function of the circulation with exercise at 3 G was met by considerably smaller increments in both a—$\bar{v}$ O_2 difference and heart rate than at normal gravity Since the cardiac output was initially reduced the graph expresses the fact that the cardiac output remained lower for both work loads at 3 G than at normal gravity As in the resting condition the oxygen pulse differed relatively little in magnitude at 1 G and 3 G its value being lower at 3 G by 4 and 9 per cent at the two work loads respectively

That the delivery of oxygen to the working muscles at 3 G was largely adequate is indicated by the fact that the oxygen uptake increased by about the same rate with a change from 300 kpm/min to 600 kpm min as it did at normal gravity (*cf* Table II)

From the reductions observed in $BHCO_3$ at 3 G with increasing work loads (Table III) it can be calculated that the arterial lactate concentration had increased by about 1 0 mM liter at 300 kpm min and by 2 3 mM/liter at 600 kpm min These calculations were based on the assumption that four fifth of the decrease in bicarbonate is accounted for by increase in lactic acid (*cf* De Lanne Barnes and Brouha 1959) and by using a conversion factor of 1 28 for obtaining the lactate concentration in arterial blood from plasma

oncentration (*cf* Grodins 1950) The calculated increments in lactate con entration values tally not only with those obtained in a previous study wher lood lactates were measured following exercise a 3 G under similar cond ons Rosenhamer 1967), but also with those obtaining at a given pulse rate during ork under normal gravity conditions (*cf* Holmgren and Ström 1959) They hus support the conclusion that the oxygen transport to the working muscles t 3 G was comparable to that with submaximal work loads at normal gravity

nfluence of Increased G on Pulmonary Function

fficiency of Ventilation

The observations that the physiological dead space to tidal volume ratio ncreased and the effective alveolar to total ventilation ratio decreased follow *ng the change from 1 G to 3 G both at rest and during leg exercise (II),* demonstrate that increased G load in the head seat direction cause tion of the net efficiency of ventilation With exercise at 1 G and 3 G the physiol ogical dead space to tidal volume ratio decreased, and the eff ve alveolar o total ventilation ratio increased, indicating a higher overall pulmonary gas exchange efficiency during exercise, not only at normal gravity but also under the influence of increased G

Both at rest and during exercise the mean values for V_E and Eff V_A were higher at 3 G than at 1 G However, the higher Eff V_A values at 3 G can be

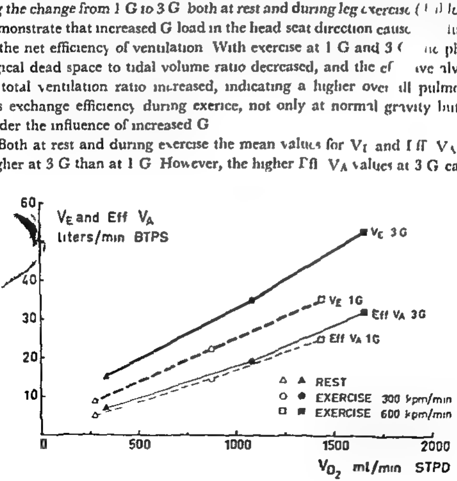


Fig 11 Respiratory minute volume V_E and effective alveolar ventilation (Eff V_A) functions of oxygen uptake V_{O_2} during rest and exercise at 1 G and 3 G (based group means from Table III n = 8

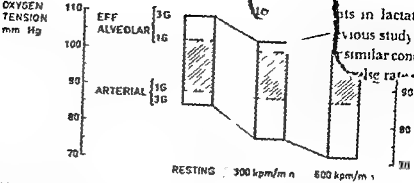


Fig 12 Alveolar arterial oxygen differences at rest and at two levels of exercise Total height of columns represents difference at 3 G height of hatched areas represents difference at 1 G Mean values of 8 subjects

Lilienthal *et al* (1946) and Filley, Gregoire and Wright (19 4), on the other hand have reported significant 8—10 mm Hg increment of the A—a O_2 difference in moderately strenuous exercise In contrast to the results obtained at 1 G the calculated mean A—a O_2 difference was und to increase with the work load when the subjects were exposed to 3 Fig 12) Fig 12 also illustrates that the fall observed in arterial P_O with exercise can mainly be ascribed to this increment of the A—a O_2 difference

In the normal subject breathing room air at sea le e transmembrane gradient for oxygen in the lungs is negligible both t and during sub maximal levels of exercise (Turino *et al* 1963 Stau 1963) Assuming this to be true also at 3 G most of the changes observed in the A—a O_2 difference with 3 G rest and exercise must have been caused by changes in either the true veno arterial shunt component or in the virtual shunt component created by uneven distribution of alveolar ventilation to pulmonary capillary flow or else by changes in both types of shunts Since the present data were obtained from air breathing experiments only they do not permit separation of true veno-arterial hunting from the unequal V_A/Q_c effect However the changes in total venous admixture (Q_s) have been calculated (Fig 13) not only in terms of (A) percentage of cardiac output (Q_s/Q %) but also in terms of (*B*) milliliter mixed venous blood shunted per minute (Q_s ml/min) (*C*) arterial oxygen deficit expressed as vol % O_2 (C_c-C_a vol % O_2) and (*D*) arterial oxygen deficit expressed as milliliter O_2 per minute (Q / [C_c C_a] ml O_2/min)

With exposure to 3 G at rest Q_s Q and Q_s (ml/min) remained essentially unchanged, which is at variance with earlier observations of Barr (1963) who found Q_s Q to be markedly increased at 5 G and those of Bjurstedt *et al* (1962) who noticed a slight increase of the Q_s/Q ratio on shifting from the supine to the erect posture A possible explanation for the apparent discrepancy

almost quantitativel the [illegible] investigations may be as follows In studies hence, the physiolog[illegible] pu[illegible]monary blood flow at different magnitudes of G what higher at 3 G head seat direction Bryan *et al* (1965) found a marked plotted against [illegible] the dependent regions of the lungs when the effective force very increased from zero to 1 G (= change from supine to upright position) [illegible] beyond the 1 G level the flow changed only little indicating that the basal vessels were then close to the limit of their distensibility It might then be expected that in so far as uneven distribution of perfusion in the dependent regions of the lungs accounts for the venous admixture effect, an increase of the G force beyond 1 G would cause only a slight increase in Q_s/Q It might further be expected that this slight increase in Q_s/Q would be cancelled by concomitant changes in the distribution of ventilation In the upright position not only the perfusion but also the ventilation per unit lung volume is greater in the lower than the upper portions of the lung (Ball *et al* 1962 Bryan *et al* 1964), and this difference increases with headward acceleration (Glaister 1965 Bryan Milic Emili and Pengelly 1966) If then under resting conditions the ventilation gradient down the lung increases somewhat more than the perfusion gradient it would explain the present observation that Q_s/Q and Q_s were unaffected by exposure to 3 G in the resting condition At still higher G levels on the other hand the intravascular pressure at the base of the lung must become very high and some extravasation of fluid seems probable (Bryan *et al* 1965) Alveolar membrane edema and basal lung collapse would then ensue, resulting in rapidly increasing shunt values as was observed by Barr (1963) in his experiments at 5 G

At normal G the Q_s/Q ratio was found to decrease with light and moderate exercise which is in agreement with the results reported by Bartels *et al* (1955) and Hesser and Matell (1965) As can be seen in Fig 13 *D* the calculated arterial O_2 deficit in ml O_2 per minute increased with the rate of O_2 uptake and hence with the work load whereas the same O_2 deficit when expressed in vol % O_2 (Fig 13 *C*) showed a slight increment only at the heavier work load (Table IV) Asmussen and Nielsen (1960) and Hesser and Matell (1965) have also noticed that the O_2 deficit in ml O_2 per minute increases with the rate of O_2 uptake While Asmussen and Nielsen found the O_2 deficit in vol % O_2 to increase with the work load Hesser and Matell observed slight decrements in this variable during light and moderate exercise Indirect evidence was presented by Hesser and Matell (1965) that the decrease in Q_S/Q occurring in response to light and moderate exercise at normal G is mainly due to an improved ventilation/perfusion distribution created by a reduction of the number of alveoli with low V_A/Q ratios whereas changes in the anatomical shunt component are of minor importance

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Aars H (Institute of Experimental Medical Research University of Oslo Norway) THE RE-SETTING OF AORTIC NERVE ACTIVITY IN EXPERIMENTAL HYPERTENSION

The re setting of arterial baroreceptor activity in hypertension is well established although mainly based on studies of the carotid sinus nerve Changes in the vessel wall have been suggested as one of its causative factors

In the present study rabbits were made hypertensive by unilateral silk wrapping of the kidneys followed after four weeks by contralateral nephrectomy Electroneurograms of the aortic nerves were obtained 3—15 days after the nephrectomy when the mean blood pressure of the six rabbits was 205/160 mm Hg The electrical impulses were rectified and integrated allowing a quantitative study of the nerve activity at various blood pressure levels The recordings with intact circulation showed a marked re setting The same firing rate as in normals was in hypertensive rabbits found at much higher pressures Similar changes were found in perfusion studies

Subsequent in vitro tension/length studies of circular aortic strips from the receptor areas gave roughly normal results and failed to explain the re setting of the nerve activity Possible mechanisms of re setting will be discussed

Ahtee L. and M K. Paasonen (Department of Pharmacology University of Helsinki Finland) RELEASE OF 5-HYDROXYTRYPTAMINE FROM BLOOD PLATELETS BY CHLORPROMAZINE IMIPRAMINE AMITRIPTYLINE AND THEIR DESMONOMETHYL DERIVATIVES

There are differences both in the uptake of certain phenothiazines and related agents by platelets and red cells as well as in their ability to release 5HT from platelets and to cause haemolysis (Ahtee and Paasonen 1965 1966) The conversion of the tertiary dimethylamine group in these drugs to a secondary monomethylamine group is known to increase their central action

Arterial blood drawn from rabbits under ether anaesthesia was mixed with EDTA and the platelet rich plasma was incubated in air at 37°C by gentle shaking Platelet 5HT was estimated spectrophotofluormetrically The demethylated derivatives of chlorpromazine imipramine and amitriptyline released more 5HT from platelets than the parent compounds did In 4 to 5 experiments during 3-hr incubation at 10^{-4}M concentrations chlorpromazine released 16±4 (S E) imipramine 17±5 and amitriptyline 30±3 % of the platelet 5HT The corresponding values for desmonomethyl chlorpromazine were 60±8 for desipramine 63±12 and for nortriptyline 70±8 % At a concentration of 1.5×10^{-4}M desmonomethylchlorpromazine released slightly more 5HT than 3×10^{-4}M concentration of chlorpromazine

The absorption of chlorpromazine and desmonomethylchlorpromazine into platelets was studied spectrophotometrically after 1 hr incubation of platelet rich plasma with these agents Platelets took up about twice as much desmonomethylchlorpromazine as chlorpromazine This difference corresponds to the 5HT releasing ability of these compounds The ability of the drugs to lower surface tension of saline was similar The different affinities for fat and different partition coefficients for the agents will be discussed (Supported by USPHS Grant MH 5832)

Ahtee L and M K Paasonen, *Ann Med exp Fenn* 1965 43 101—105 and *J Pharm Pharmacol* 1966 18 126—128

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Aitio A and O Hanninen (Department of Physiology University of Turku Finland) THE EFFECT OF CINCHOPHEN ADMINISTRATION ON THE TRANSAMINASE ACTIVITY OF RAT LIVER

The peroral administration of cinchophen causes a high increase in the activity of some enzymes in the glucuronic acid pathway On the other hand it causes an excessive loss of carbohydrates as ascorbic and glucuronic acids into urine Transaminases provide aminoacids for protein synthesis and ketoacids for gluconeogenesis

The activity of rat liver tyrosine 2-oxoglutarate transaminase was found to be almost double after three or six peroral administrations of one millimole of cinchophen compared with the controls both 24 and 48 hours after the last administration On the other hand alanine 2-oxoglutarate transaminase activity remained unchanged after three doses of the drug and the aspartate 2-oxoglutarate transaminase after six doses Tyrosine 2-oxoglutarate transaminase is a soluble enzyme alanine 2-oxoglutarate transaminase was analyzed from the postmitochondrial supernatant fraction and aspartate 2-oxoglutarate transaminase from isolated mitochondria

Cinchophen and its derivatives have been found to be powerful inhibitors of alanine and aspartate 2-oxoglutarate transaminases *in vitro* (Hanninen and Hartiala 1965) The tyrosine 2-oxoglutarate transaminase is not however inhibited by the drugs

Hanninen O and K Hartiala Inhibition of transaminases by cinchophen and its derivatives *Biochem Pharmacol* 1965.14 1073

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Andén N E (Department of Pharmacology University of Goteborg Sweden) EFFECT OF SYNTHESIS INHIBITION AND RESERPINE ON THE MONOAMINE LEVELS IN DIFFERENT PARTS OF THE CENTRAL NERVOUS SYSTEM

The tissue monoamine levels can be reduced in mainly two different ways by inhibition of the synthesis and by interference with the storage The importance of the nervous impulse flow for the amine disappearance was studied in acutely transected spinal cords All the monoamines in the spinal cord are present in nerve fibres descending from the lower brain stem The degeneration of these fibres caudal to a lesion starts only 3 days after the operation In a sectioned cord the lowering of the noradrenaline in the caudal part after inhibition of the tyrosine hydroxylase by alpha methyltyrosine methylester was almost completely prevented On the other hand after reserpine treatment the disappearance of the noradrenaline caudal to a section was almost of the same magnitude as cranial to it Similar differences were found between the effects of tryptophan hydroxylase inhibitors and reserpine on the 5-hydroxytryptamine cranial and caudal to a spinal cord lesion

In the telencephalon and diencephalon inhibition of the tyrosine hydroxylase produced a catecholamine reduction which in per cent was approximately equal in the different parts After treatment with reserpine in a small dose large regional differences were observed The noradrenaline in e g the neocortex was much more lowered than in e g the hypothalamus Reserpine also reduced the noradrenaline more than the 5-hydroxytryptamine These results may indicate that the impulse flow of the monoamine nerves is about the same in the different parts of the central nervous system and that the regional differences after reserpine treatment can only partly be explained by differences in nervous activity

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Akre E and H Bugge-Asperheim (Institute for Experimental Medic Research University of Oslo Ullevål Hospital Oslo Norway) TH EFFECT OF HEART RATE ON THE MYOCARDIAL CONTRACTILIT AND MYOCARDIAL OXYGEN CONSUMPTION

The effect of changes in heart rate (HR) on myocardial contracti state and myocardial oxygen consumption (MVO_2) has been studied intact dogs anesthetized with chloralose urethane

HR was increased in steps with an artificial pacemaker and an electrod in the right atrium Myocardial blood flow (MF) was estimated fro hydrogen desaturation curves in the coronary sinus MVO_2 was calculate from MF and the arterio-venous oxygen difference Left ventricular pre sure maximum rate of rise in the left ventricular pulse pressure (dp/d were measured Thermal indicator dilution technique was used for th estimation of the cardiac output (CO)

With increasing heart rates there was an almost linear increase dp/dt

MVO_2 increased with elevated HR

At a fixed HR infusion of noradrenaline (NA) increased dp/dt an MVO During NA infusion increase in HR led to no increase or only slight increase in dp/dt When a β blocking agent propranolol was give at a fixed HR there came a marked decrease in dp/dt and MVO_2 but in crease in HR led to a linear increase in dp/dt

There was a good correlation between MVO_2 and the product of dp/c and HR

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Andersen, H , L. Korner S Landgren and H Silfvenius (Department of Physiology University of Goteborg Sweden) FIBRE COMPONENTS AND CORTICAL PROJECTIONS OF THE ELBOW JOINT NERVE IN THE CAT

The projection of knee joint afferents to the central nervous system has been studied in several investigations The information obtained is useful in attempts to interpret the function of central mechanisms The joint afferents from the forelimb has so far not been utilized mainly because of the difficulties involved in the dissection and indentification of a pure forelimb joint nerve

The present report describes peripheral and central responses to stimulation of the elbow joint branch of nervus musculocutaneus The elbow joint nerve branches off from n musculocutaneus at the level of foramen supracondylicum of the humerus It can be separated from the branches to m brachialis and the cutaneous bundle of the nerve which proceeds to the lower arm The branch was identified by dissection under the microscope to its ramifications in the joint capsula and also by recording of the impulse discharge in the nerve evoked by joint movements and by localized pressure on the joint capsula

The action potential recorded in the musculocutaneous nerve in response to electrical stimulation of the joint branch showed three components The thresholds of these were 1 1.5 and 2 times threshold of the response to joint nerve stimulation The mean conduction velocities of the fastest fibres in the groups were 83 57 and 42 m/sec

Electrical stimulation of the joint nerve evoked an initially positive cortical surface potential in the region of the postcruciate dimple mean latency 9 msec) Initially positive (or negative) surface potentials were also recorded laterally (12 msec) and rostrally (26 msec) to sulcus cruciatus In the S I and S II forelimb areas small initially positive potentials (9 and 11 msec) were observed The thresholds of the cortical responses were I—II times that of the afferent volley It was often necessary to use repetitive stimulation in order to evoke them

Andersen H T and B.-E Hustvedt (Institute of Nutrition Research and Institute of Zoophysiology University of Oslo Blindern Norway) THE EFFECT OF CARBON ANHYDRASE INHIBITION ON ACID-BASE BALANCE AND CO TRANSPORT IN THE DUCK DURING POST DIVING HYPERVENTILATION

Carbonic anhydrase is usually present in excess of the amount needed to handle normal CO transport It is of interest therefore to study the importance of carbonic anhydrase in a system in which the enzyme substrate (CO_2) is elevated so as to put an increased demand on the transport mechanisms for CO One way in which to achieve such an experimental situation would be to aphyxiate the subjects until they enter a condition of combined respiratory and metabolic acidosis Since most warm blooded animals do not tolerate severe asphyxia we have chosen to study an avian diver the domestic duck which may remain submerged for as long as 15 min The normal acid base pattern during diving has been previously investigated in this species (Andersen *et al* 1965)

Acetazolamide (Diamox®) was administered intravenously in doses of 10—100 mg/kg body weight The smallest dose of acetazolamide required *for maximal inhibition of blood carbonic anhydrase in the duck is* approximately 50 mg/kg body weight Inhibition of carbonic anhydrase produced no decrease in total CO of arterial plasma in the pre-diving period despite acidosis and hyperventilation (pH 7.25)

Asphyxia and severe acidosis were induced in diving experiments of 6 min duration Total-CO in arterial plasma and pH in arterial blood were determined before during and after the dive Inhibition of carbonic anhydrase caused a reduced buffering capacity in the blood This was particularly noticeable in the post-driving period during which the arterial *pH decreased to about 6 95 (normal value 7 10—7 15) The excretion of CO* due to post-diving hyperventilation reduced the arterial total-CO_2 from approximately 25 mEq/l to approximately 15—20 mEq/l whereas it normally falls to about 9—10 mEq/l upon emersion

Andersen H T B E Hustvedt and A. Lovo Acid Base Changes in Diving Ducks *Acta physiol scand* 1965.63 128—132

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Andersen H T and A Lovo (Institute of Nutrition Research and Institute of Zoophysiology University of Oslo Blindern Norway) HYPOXEMIA AND CONSCIOUSNESS IN THE DUCK DURING PROLONGED DIVING

Diving vertebrates exhibit a series of cardiovascular adjustments upon ibmersion Most organs and tissues are shunted out of the systemic rculation in fact the animals turn themselves into a heart lung brain eparations This explains how the divers conserve their limited O_2 de)ts in order to remain under water for extended periods of time with no gns of motor disturbances or other symptoms of organic injury How er it is quite clear that the diving vertebrates nevertheless endure ex eme hypoxemia during prolonged submersions Blood analyses have iown that the arterial blood of homoiothermic divers seals ducks and enguins may contain less than 3 volumes per cent of O_2 towards the end a maximal dive (Sholander 1940) and in poikilothermic divers the O_2 .posits may be virtually depleted during a prolonged submergence (An ersen 1961) Since diving animals do not lose consciousness during such tended underwater exposures although their stores of O_2 may be nearly thausted it would be of interest to investigate the mechanism which lows the central nervous system to function properly at such a very w content of O_2 in the blood

Oxygen dissociation curves of duck blood have been constructed *in tro* for various values of pH which are experienced by the birds during ving Using the ducks themselves as living tonometers the *in vivo* dis iation curves for O_2 were superimposed on those previously constructed he HbO_2 in the arterial blood sometimes fell to 3—5 % during prolonged eriods of underwater exposure However the dissociation curve of duck lood is shifted much downwards and to the right under such conditions herefore the tension of any O_2 present in the blood is relatively high lready 5 % HbO_2 corresponds to a pO_2 of 30 mm Hg This explains how ucks are able to utilize their O_2 deposits fully and remain conscious iroughout a prolonged dive

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Andersen H T and A Løvø (Institute of Nutrition Research and Institute of Zoophysiology University of Oslo Blindern Norway) RESPIRATORY EFFECTS OF HYPERCAPNIA AND HYPOXEMIA IN AVIAN DIVERS (DUCKS)

Diving vertebrates are able to remain under water for extended periods of time and endure a considerable degree of asphyxia It is commonly assumed therefore that accumulation of CO_2 and lack of O_2 are relatively inefficient in stimulating respiration in diving animals It has been reported that the respiration of ducks is actually inhibited when they inhale gas mixtures containing large amounts of CO_2 (Orr and Watson 1913 Hiestand and Randall 1941) These reports have led later authors to believe that CO_2 normally acts as a respiratory inhibitor in diving birds (Salt and Zeuthen 1960) However ducks do not experience CO_2 concentrations of 10 volumes per cent or more in their lungs and air sacs even during very prolonged dives (Andersen 1959)

It is not likely that CO_2 acts as a respiratory stimulus in certain animals but inhibits respiration in others We have reinvestigated the respiratory effects of hypercapnia hypoxemia and asphyxia in the duck and found that high CO_2 as well as low O_2 concentrations are very potent respiratory stimuli within physiological limits CO_2 increases the respiratory minute volume because it causes a large increase in the tidal volume whereas lack of O_2 results in a corresponding augmentation of the respiratory minute volume due to acceleration of the respiratory frequency When ducks breathed gas mixtures which contained O_2 and CO_2 in approximately the same proportions as are found in their lungs and air sacs towards the end of a long dive the respiratory minute volume increased to approximately three times the resting level Thus apnoea during diving takes place not because hypercapnia and hypoxemia develop but in spite of CO_2 accumulation and lack of O_2

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Andersen P K Junge and O Sveen (Laboratory of Neurophysiology Institute of Anatomy University of Oslo Norway) CORTICO THALAMIC FACILITATION OF CUTANEOUS SENSORY INFORMATION

Central control of afferent sensory information is exerted either on the sense organ itself or as an inhibition of the synaptic transmission along a sensory pathway The present report describes a new effect facilitating the synaptic transmission of cutaneous impulses through the thalamus

A single shock to the arm-area of the post-cruciate cortex in the n ventralis posterolateralis (VPL) evokes a field potential consisting of an initial sharp deflection (A) followed by a negative wave (N) with small spikes superimposed and a later positive wave (P) The A deflection represents the antidromic invasion of thalamo-cortical relay cells The N wave is due to a transsynaptic activation of thalamic cells either through axon collaterals of the thalamo-cortical axons or through cortico-thalamic fibres The P wave is due to the large recurrent IPSPs of thalamic relay cells (Andersen and Eccles 1962) Following repetitive stimulation of the post-cruciate gyrus (10—20/sec) the N wave increased greatly and finally abolished the P wave The cortical stimulus then activated the relay cells both anti and orthodromically The corticothalamic depolarizing effect on relay cells was also demonstrated by a reduction of the spike latency and an increase of the number of spikes in response to stimulation of cutaneous foreleg nerves The effect is optimal at a stimulus rate of 15—20/sec and is absent below 5/sec and above 50/sec

The mechanism is somato-topically organized stimulation of the hindleg area having little effect on forelimb relay cells

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Andersen P and J C Eccles *Nature (Lond)* 1962.196 645—647

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Andersson B., M Jobin and K. Olsson (Department of Physiology Veterinarhogskolan Stockholm Sweden) THIRST AND INCREASED SALT EXCRETION INDUCED BY RAISING THE NaCl-CONCENTRATION IN THE 3rd BRAIN VENTRICLE

Injections of 0 1 ml of 0 85 M NaCl were made at 30 min intervals into the 3rd or the lateral brain ventricle of unanaesthetized goats As could be expected from previous experiments in the same species (Andersson 1953) each injection of NaCl solution into the 3rd brain ventricle induced a strong urge to drink Thirst became apparent within a minute and had disappeared again after 20 min This effect was not obtained in the hydrated animal Similar injections of hypertonic NaCl solution into the lateral brain ventricle had no effect or a much weaker and more delayed thirst eliciting effect Injections of 0 1 ml of 0 85 M NH Cl into the 3rd ventricle did not induce any urge to drink in the goats

During the periods of repeated injections of 0 85 M NaCl into the 3rd ventricle the urinary excretion of Na and Cl increased by 5 to 10 times and the K excretion was approximately doubled At the same time urine flow increased about 3 times The period of increased urinary salt excretion was followed by a second increase in urine flow (up to 7 times the basic flow) This second increase in urine flow had the character of a water diuresis (a return of salt excretion to or below basic level and a fall in urinary electrolyte concentration to a very low level) Administration of aldosterone did not prevent those effects of hypertonic NaCl

Repeated injections of 0 1 ml of 0 85 M NaCl into the lateral brain ventricle or of 0 85 M NH Cl into the 3rd brain ventricle were not seen to cause an increase in urinary salt excretion

Andersson B The effects of injections of hypertonic NaCl solutions into different parts of the hypothalamus of goats *Acta physiol scand* 1953 *28* 188—201

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Andersson S A (Department of Physiology University of Goteborg Sweden) SUPPRESSION OF CORTICAL SPONTANEOUS BARBITURATE SPINDLES VIA SPINAL PATHWAYS

The effect of peripheral stimulation on cortical spontaneous barbiturate spindles was investigated in cats anaesthetized with pentobarbital sodium Selective lesions in the spinal cord permitted a study of the cortical effects mediated by different spinal pathways

The most potent suppression of the spindle activity was obtained by a pathway in the ventral funiculus identified as the bilateral ventral flexion reflex tract (bVFRT) by Lundberg and Oscarsson (1962) Via this pathway a longlasting and strong generalized abolition of the spindles was elicited especially when the tonic inhibition of the transmission from the flexion reflex afferents (FRA) to this pathway was removed (Holmqvist Lundberg and Oscarsson 1960)

Suppression of the cortical spindles could also be obtained via pathways in the dorsolateral funiculus In part this effect was mediated by the spino-cervico-lemniscal pathway (SCL) (Andersson 1962) Via this pathway a change in the slow wave pattern was obtained in the somatosensory receiving areas in response to light cutaneous stimuli Strong stimuli such as pinching a limb gave a generalized abolition of the cortical spindles A generalized cortical effect was also obtained by another unknown pathway in the dorsolateral funiculus This pathway received a bilateral input in the spinal cord and was most effectively activated by pinching the limbs

The effect on cortical spindles mediated via the dorsal column lemniscal pathway was mainly localized to the somatosensory projection areas However in some animals a clear but shortlasting generalized suppression of the barbiturate spindles was obtained also via this pathway

Andersson S A *Acta physiol scand* 1962 56 Suppl 194 74 pp
Holmqvist B A Lundberg and O Oscarsson *Arch ital Biol* 1960 98 60—80
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Appelberg B (Department of Physiology Veterinarhogskolan Stockholm Sweden) CONTROL OF MUSCLE SPINDLE DYNAMIC SENSITIVITY FROM THE MESENCEPHALON AND THE MEDULLA OBLONGATA

Repetitive electrical stimulation within a restricted region in the mesencephalon may cause a strong and quite selective increase in the dynamic sensitivity of muscle spindles in the flexor digitorum longus muscle of the cat This control system is extremely sensitive to the influence of anesthesia and the use of a volatile anesthetic such as Fluothane is to be preferred The effective stimulating region comprises at least the caudal part of the red nucleus plus an area extending like a band from the level of the red nucleus caudally and dorsally to a position just ventrally to the trochlear nucleus The descending pathway from this mesencephalic region for dynamic spindle control seems to have a relay in the medulla oblongata Micro-electrode recording from a restricted area at the level of the inferior olive revealed a focal potential evoked by single shock stimulation in the mesencephalic region described. The response consisted of a fast positive/negative/positive potential deflection followed by a slow negative wave after a latency of about 0.8 msec. On the negative wave cellular responses were frequently observed. In experiments where the medullary electrode could be used also for stimulation it was found that repetitive stimulation within a region which coincided well with the on responding to mesencephalic stimulation also caused an increase in muscle spindle dynamic sensitivity The medullary region for control of muscle spindle dynamic sensitivity is not yet histologically localized. It seems reasonable to suggest that the occurrence of such a medullary synaptic relay on the pathway from the mesencephalon to the dynamic fusimotor neurones may serve as an explanation of the great anesthesia sensitivity of this control system

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Aquilonius S-M and ■ Winbladh (Forsvarets Forskningsanstalt Sundbyberg Sweden) EFFECT OF CERTAIN DRUGS ON CEREBRAL OXYGEN AVAILABILITY IN ANAESTHETIZED AND CONSCIOUS RABBITS

The used method only allowed measurement of relative changes in oxygen concentration at the electrode tip oxygen availability

Open-ended polarographic electrodes (a 30 μ platinum wire insulated in a 100—150 μ glass capillary) were implanted deep in the sensory cortex or the underlying white matter of rabbit brain and fixed to the skull with dental cement The reference electrode consisted of ■ one mm silver wire implanted ■ the ear The electrode was polarized with a constant voltage of —0 68 volts

Acutely implanted electrodes were used in experiments with anaesthetized rabbits In these experiments femoral blood pressure and respiration were also recorded The function of the electrode was tested by breathing ■ gas mixture consisting of 5 % O_2 95 % N_2 and 6.5 % CO_2 93.5 % O_2 respectively The changes in oxygen availability thus caused by hypoxia and hyperoxia were the same before and after drug administration

Following administration of acetylcholine and nor-epinephrine a close correlation was found between the changes in oxygen availability and blood pressure The same correlation was found following injection of the cholinesterase inhibitors physostigmine and 23 S-N [Diethoxy (2-dimethyl aminoethylthio) phosphine oxide] except when the respiration had been severely depressed for several minutes when the oxygen availability decreased in spite of an elevated blood pressure When atropine was given after cessation of respiration blood pressure and oxygen availability increased simultaneously The early decrease in periferal arterial oxygen saturation reported by Fredriksson *et al* (1960) as the first symptom of poisoning with cholinesterase inhibitors was not reflected in an early decrease in brain oxygen availability

In conscious rabbits with permanently implanted electrodes the changes in oxygen availability were the same as in the anaesthetized rabbits with acutely implanted electrodes

Fredriksson T C H Hansson and B Holmstedt *Arch int Pharmacodyn* 1960 *126* 288

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Arstila A and J Wersall (Department of Anatomy University of Turku and Department of Otolaryngology and King Gustav V Research Institute Karolinska Sjukhuset Stockholm Sweden) ULTRA STRUCTURAL CHANGES IN THE OLFACTORY MUCOSA OF THE GUINEA PIG AFTER NEOMYCIN ADMINISTRATION

Numerous experimental investigations have been published of the toxic effects of various antibiotics of Streptomyces group on the cochlear sensory cells Clinical cases have been shown that Neomycin might have toxic effect on the olfactory mucosa but no experimental evidence is shown in order to verify this

In this study Neomycin was given to guinea pigs both intraperitoneally for six days and locally for 21 consecutive days

After intraperitoneal administration most changes were seen in the basal area of the epithelium near the underlying capillaries Especially in the supporting cells marked changes were seen consisting of accumulation of lysosomes myelin figures small vesicles and large whorls of double membranes as well as the degeneration of mitochondria In the sensory cells degeneration of mitochondria and the increase in the number of *lysosomes and basal bodies were observed*

After local administration extensive changes were seen near the free border of the epithelium A vast increase in the number of mitochondria ribosomes double membranes and lysosomes were seen in the supporting cells as well as the lengthening of the microvilli of them Degeneration was seen in the outer membranes and vesicles of the cilia in the terminal swellings of the sensory cells In many cases they were also replaced by microvilli Also degeneration of mitochondria and the occurence of lysosomes in them were observed

Neomycin is known to effect the protein synthesis of the sensory cells and to have an inhibitory effect on the synaptic transmission The changes in the supporting cells suggest that Neomycin first effects the metabolism of these cells The changes in the cilia of the sensory cells indicates that Neomycin might inhibit the depolarisation of the ciliar membranes now adays regarded to be the primary sites for the olfactory sensation The changes in the structures of the mitochondria and lysosomes of these cells on the other hand may be signs of more pronounced and permanent degeneration of these cells

These morphological findings suggest that Neomycin has a toxic effect on the olfaction similar to that seen in the cochlea but this must be con firmed by electrophysiological methods

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Arvidsson J, I Jurna and G Steg (Department of Physiology University of Goteborg Sweden) DESCENDING PATHWAYS MEDIATING RESERPINE RIGIDITY

The rigidity induced in rats by reserpine and other drugs inhibiting monoaminergic transmission is abolished after section of the spinal cord. The rigidity is decreased neither when the pyramidal tract is interrupted by section of the pyramids nor when the ventral quadrants of the cord are completely transected. Unilateral section of the dorsal part of the lateral funiculus abolished the rigidity on the ipsilateral side. The work on identifying structures mediating the rigidity is continued.

Arvill A and K. Ahrén (Department of Physiology University of Goteborg Sweden) EFFECTS OF INSULIN ON AMINO ACID TRANSPORT AND PROTEIN BIOSYNTHESIS IN THE ISOLATED INTACT LEVATOR ANI MUSCLE FROM THE RAT

The transport over the cell membrane and the incorporation into muscle protein of various normal and non utilizable amino acids has been studied in a new intact mammalian muscle preparation namely the levator ani muscle of immature male rats It has earlier been shown that this muscle can be dissected out and incubated with undamaged cell membranes (Arvill and Ahrén 1965 1966)

Insulin added to the incubation medium stimulated the transport of the non utilizable amino acid α amino-iso-butyric acid (AIB) The minimal concentration of insulin required for this effect was 10^{-5} IU/ml and a maximal effect was seen with 10^{-3} IU/ml The dose response curve was slightly different during the seasons of the year

The rate of transport was determined with various concentrations of AIB and the results have been expressed in terms of Michaelis—Menten kinetics

Insulin had an effect on the rate of uptake of AIB also when the protein synthesis was blocked by puromycin and when RNA synthesis was blocked by actinomycin D

Insulin stimulated also the incorporation of glycine H^3 L-valine-C and leucine-C into the muscle protein while an increased accumulation in the intracellular water in the presence of insulin could be seen only with glycine H^3 When the protein synthesis was blocked with puromycin an effect of insulin on the transport was seen also with leucine-C The last mentioned observation is in agreement with experiments on the diaphragm and heart muscle (e g Wool 1965)

Arvill A and K Ahrén, *Nature (Lond)* 1965.206.309
Arvill A and K Ahren *Acta endocr (Kbh)* 1966.52 325
Wool I G *Fed Proc* 1965 24 1060

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Aukland K (Institute for Experimental Medical Research University of Oslo Ullevål Hospital Oslo Norway) HEAT CLEARANCE IN THE RENAL MEDULLA

Heat clearance from a tissue is generally considered to be determined by its blood flow and thereby forms the basis for various methods for local blood flow measurements However a critical appraisal of the mechanism of heat clearance in the renal medulla seems necessary because of its unique system of long looped capillaries the *vasa recta* These vessels act as a passive exchange counter current system which impede the entrance and removal of diffusible substances from the medulla by the blood stream

Experiments were performed on dog kidneys Thermocouples made from 0 1 mm thick copper and constantan wires were stitched through the kidney and fixed in various positions in the medulla A thermocouple in the aorta served as reference Sudden changes in renal arterial temperature were produced by infusion of 0 9 % saline of room temperature at a rate of 2—5 ml/min The rate of heat dissipation — and heat uptake after stopping the infusion — were recorded as the rate of temperature change to the new level After a delay of 10—60 seconds the curves approached the new equilibrium exponentially with an average rate constant of 0,58 min^{-1} in the inner medulla This is about ten times faster than the clearance of hydrogen gas suggesting that heat clearance is not a function of *vasa recta* flow Reduction of renal arterial perfusion pressure from 120 to 30 mm Hg reduced heat clearance by only 10—20 % Increasing urine flow from 0 1 to 5 0 ml/min increased heat clearance by 30 %

It is concluded that heat is removed from the inner medulla mainly by conduction (diffusion) to the cortex and the great vessels at the corticomedullary junction and is largely independent of *vasa recta* flow The conclusion is supported by a calculated value for inner medullary heat clearance by conduction alone of about 0 4 min^{-1}

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Axelsson J., B Holmberg and G Hogberg (Department of Zoophysiology University of Goteborg Sweden and Department of Physiology University of Iceland Reykjavik Iceland) ATP AND INTESTINAL SMOOTH MUSCLE

ATP applied extracellularly relaxed spontaneously maintained tone and contractures of taenia coli from the guinea pig (Axelsson Holmberg and Hogberg 1965) The abolition of spontaneously maintained tone is secondary to inhibition of spike discharge and hyperpolarization High potassium contractures are relaxed without significant changes in membrane potential In the case of drug induced contractures ATP prevents or shortens the initial depolarization produced by acetylcholin and carbachol and shortens the duration of mechanical response Liberation of inorganic phosphate from ATP applied to the bathing solution is considerably greater than can be accounted for by spontaneous hydrolysis This phosphate liberation is presumably due to unspecific phosphatase activity as it was not significantly decreased by g strophantine or parachloromercuribenzoate These inhibitors caused initial increase in the frequency of discharge but did not abolish the inhibitory action of ATP In calcium free solution ATP failed to abolish spike discharge AMP and adenosin have the same effects as ATP GMP and guanosine have no effect

All these observations are consistent with the view that the effects of extracellularly applied ATP on spontaneous activity contractures and responses to drugs are through interferences with membrane permeabilities This effect may be confined to the adenosine part of the molecule and not to increased energy supply Tracer experiments indicate that adenosine is taken up by the muscle therefore an intracellular action can t be excluded

Axelsson J B Holmberg and G Hogberg Some effects of ATP and adrenaline on an intestinal smooth muscle *Life Sciences* 1965 4 817—821

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Axelsson, J, B Johansson and O Jonsson (Department of Physiology University of Goteborg Sweden) ON THE MECHANISM OF BETA ADRENERGIC ACTION ON VASCULAR SMOOTH MUSCLE

The inhibitory action of adrenergic agents on intestinal smooth muscle has been ascribed to inhibition of spike discharge and membrane hyper polarization as demonstrated in taenia coli The present experiments indicate that other mechanisms may be responsible for beta adrenergic inhibition in vascular smooth muscle

Isolated strips of rat portal vein were mounted in the muscle bath and their spontaneous phasic contractions recorded isometrically Nor adrenaline in concentrations of 1—1000 γ/litre increases both frequency and amplitude of these contractions Isoproterenol in the same concentrations causes an inhibition of the mechanical activity which appears as a reduction in the amplitude of the phasic contractions while their frequency is maintained or more often increased The association between mechanical and electrical activity in the portal vein is well documented and the increased frequency of phasic contractions can be taken as an indicator of an increased generation of conducted action potentials Therefore the pattern of the isoproterenol responses indicates that the beta adrenergic inhibition on this vascular smooth muscle is not simply due to inhibition of spike discharge It could also be shown that the reduction in the amplitude of the phasic contractions produced by isoproterenol was not the result of impaired intercellular conduction Further isoproterenol relaxed portal vein strips which were in potassium contrac ture The effects of nor adrenaline and isoproterenol on the portal vein were selectively blocked by dibenzyline and propranolol respectively

The results suggest that beta adrenergic action involves a positive chronotropic effect a largely unchanged intercellular propagation and a negative inotropic effect on the contractile machinery of the vascular smooth muscle cell

Axelsson J B Johansson and O Jonsson (Department of Physiology University of Goteborg Sweden and Department of Physiology University of Iceland Reykjavik Iceland) SIMULTANEOUS RECORDING OF ELECTRICAL AND MECHANICAL ACTIVITY OF VASCULAR SMOOTH MUSCLE USING THE SUCROSE-GAP TECHNIQUE

The portal vein of the rat or longitudinal strips of this vessel were cut out The hepatic end of the vein was connected to a force-displacement transducer and continuously superfused with Krebs-solution The mesenteric end was anchored at a fixed point and depolarized by Krebs solution in which NaCl was replaced by KCl The section of the preparation between the recording electrodes was superfused with isotonic sucrose solution

This technique originally described by Stampfli (1954) permits extracellular recording of relative potential changes The records of the membrane potential reflect the sum of electrical activity in the piece of muscle from which the mechanical activity is recorded The amplitude of spikes as well as the mechanical force are dependent on the synchrony of spontaneous discharges This provides favourable conditions for studying the relationship of electrical to mechanical activity

At low resting tension (<100 dyn) the typical activity consisted in spontaneous bursts of spikes followed closely by increase in tension (see also Bohr and Funaki 1964) Both the duration of bursts and their interval varied at unchanged resting tension In quiesent preparation spike discharge could be initiated by increasing the resting tension and within limited range the frequency of discharge was related to the initial or resting stress

At present we have studied the effects of varying the external medium (with respect to ionic composition glucose concentration and pH) on membrane potential frequency of discharge and tension — and on the normal correlation of these parameters

Bohr D F and S Funaki *Nature (Lond)* 1964.*203* 192—194
Stampfli R *Experientia (Basel)* 1954.*10*.508—509

Bergstrom L. (*Institute of Physiology University of Helsinki Finland*)
FOETAL DEVELOPMENT OF MESENCEPHAL MOTOR FUNCTIONS IN THE GUINEA PIG

Electrical stimulation of the mesencephal brain stem has been performed *in guinea pig foetuses (age 30—65 days term 65 days) in extra uterine* placental connection with the mother animal Bipolar and unipolar steel needle electrodes insulated to the bare tip were used Square wave (50 cps 1 msec threshold and suprathreshold voltages) and sine wave pulses (50 cps threshold and suprathreshold voltages) were introduced through *the stereotactically placed electrodes The stimulated points were marked* by coagulation or iron and examined histologically The spontaneous and *reflectory motor activity of the foetus was studied before the stimulation* The motor performances were determined visually and when necessary by electromyography and motion pictures

In most of the foetuses the spontaneous activity was observed to begin (in the shoulders and forelegs) around the 40th day, and decay at a *minimum* around the 60th day (a lethargic phase) *The reflexes from the* skin of the nose and of the forelegs could be elicited from the 20th day *The trigeminal reflex which was initially irradiated to the whole upper* body was later limited to the head region

The stimulation experiments demonstrated that tonic activity of the trunk and the shoulder can be obtained from around the 20th day in early *foetuses the activity of the whole trunk is more prominent and in* later foetuses the activity of the shoulder The stimulation induced movements of the forelegs appeared on the 30th day but that of the hindlegs subsequently At the *beginning these movements were slow and tonic* and more rapid later The rhythmic continuous stepping movements appeared only *in the later foetuses (from 55 days) that is in the lethargic* phase The respiratory movements initial gasps and single thorax contractions *could be induced from the 20th day (Spontaneous appearance* only on the 30th day) These appeared in short rhythms after 50—55 days *From 55 days the respiration remained continuous after stimulation or* cutting of the umbilical cord

The results show that phases in the mesencephal motor functions of the foetal guinea pig can be differentiated in consequence of the tonicity or *phasicity or rhythmicity of the movements or the maturation of other* motor functions

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Bergstrom R M (Institute of Physiology University of Helsinki Finland)
ORGANIZATION OF DATA PROCESSING IN A PHYSIOLOGICAL LABORATORY

Data processing (DP) in physiological research generally is considered useful in 1) detection 2) collection 3) storage 4) display 5) the analysis and 6) calculation of data or 7) in control of experiments or 8) stimulation and 9) modelling physiological functions However the main principle in all these processes can be considered as 10) a mathematical mapping ($\longrightarrow$) of the studied physiological function (f_p) with a computer function (f_c) which completes the off line use of DP-equipment On lines use continues the mapping of the f_c with a new physiological function (f_p) which is in close functional connection and thus mapped with f_p This on line DP cycle

(1) $$f_p \longrightarrow f_c \longrightarrow f_p' \longrightarrow f_p$$

forms a closed feed back (mapping) loop It is well suited to the determination of transformation coefficients in the physiological process and accordingly for the study of regulatory mechanisms

For adequately effective realization of the mapping loop (1) a small digital general purpose computer is considered to be most useful the memory capacity should be at least a few thousand address locations the word length minimum ten bit and the time of execution for a memory cycle at its maximum five to ten microseconds The most important points are easy handling of the programmes a possibility for displaying of intermediary results and programme cycles and simple interface connections In a medium size physiological department it is thought useful to have the computer as a central unit connected on line with at least 3—4 laboratories through signal cables and operator lines for cooperation and signal transmission in two directions Additionally a multichannel tape unit is necessary for off line work and for the transport of data to larger computer units elsewhere The DP plan should be adapted to that of the corresponding faculty or university the connection of a large central unit to smaller laboratory computers is regarded as the solution nearest the ideal A physiological department equipped with DP tools should also make plans with a view to having engineers physicists mathematicians and programmers of its own Regular teaching in biological DP for graduates and postgraduates is considered necessary

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Bergstrom R M (Institute of Physiology University of Helsinki Finland)
TRANSFORMATION OF MECHANICAL ENERGY INTO SENSORY IMPULSES IN THE CAT SKIN

Pressures of variable complex time slopes (1—10 g; duration 0 1—1 0 sec contact surface 1 mm²) have been applied to the skin of the hind leg of the cat under light pentothal anaesthesia during registration of the impulse pattern in the activated sensory nerve fibres The time integral of the applied pressure (p) was found within certain limits to be in linear correlation with the total number (n) of the impulses in the sensory nerve fibres

(1)

$$\int_{t_1}^{t_2} p \, dt = n \, H$$

H being a constant of proportionality and t_2—t_1 the duration of the pressure pulse This result analysed by a Linc computer shows the mode of transformation of mechanical energy into sensory impulses and can be parallelled to a corresponding reverse transformation of motor impulses into mechanical energy in the striated muscle during voluntary contraction (Bergstrom 1962 a) It is put forward that a sensory motor transformation formula

(2)

$$W = \int_{t}^{t_2} E \, dt = n \, H$$

where W represents the physical enviroment of the nervous system in the form of a physical action (in Germ "Wirkung" dimension g cm² s^{-1}) E the energy of this environmnet n thae number of neural action potentials and H a neural constant applies to transformations between the neural system and its physical enviroment This transformation also capable of carrying subjective information into the human being (Bergstrom 1962 b) is brought about by the receptor and effector organs of the nervous system

This study has been supported by a grant from the Finnish State Committee of Medical Sciences

Bergstrom R M The relation between the integrated kinetic energy and the number of action potentials in the electromyogram during voluntary muscle contraction *Ann Acad Sci fenn A 5* 1962 a 94 1—24

Bergstrom R M Uber die Struktur einer Wahrnehmungssituation und uber ihr physiologisches Gegenstuck *Ann Acad Sci fenn A 5* 1962 b 94 1—23

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Bergstrom R. M K Sainio T Pitkanen and M Vehaskari (Institute of Physiology University of Helsinki Finland) THE EFFECT OF GLUTETHMIDE (DORIDEN®) ON THE EEG OF GUINEA PIG FOETUS

Glutethimide (α phenyl-α-ethyl glutarimide Doriden®) is a widely used hypnotic with a barbiturate like effect Structurally it resembles thalidomide (N phtalyl glutarimide) and it has been proposed to be a tera togenic drug

Previously the effect of several anaesthetics (Bergstrom *et al* 1966) and thalidomide (Bergstrom *et al* 1964) on the EEG of the guinea pig foetus was studied It is of interest to compare the effect of these drugs with the effect of glutethimide

The EEG was simultaneously recorded from the mother and the intra uterine foetus (Bergstrom 1962) The drug was given perorally to the mother animal

The effect of glutethimede on the foetal EEG (partly analyzed by a Linc computer) was observed to be a synchronizing one It had however some spesific effects which differed from the effects of the other drugs so far examined

This study has been supported by a grant from the Sigrid Juselius Foundation

Bergstrom R M Brain and muscle potentials from the intrauterine foetus in unnarcotized conscious animals *Nature (Lond)* 1962.195 1004—1005

Bergstrom R M L Bergstrom P Putkonen and K Sainio The effects of thalidomide on the electrical activity of the brain in the intrauterine guinea pig foetus *Med Pharmacol exp* 1964.11 119—127

Bergstrom R M D Stenberg Y Jokinen and K Jarvi The effect of anaesthetics on the electrocorticogram of the intrauterine guinea pig foetus *Med Pharmacol exp* 1966 In press

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Bie P and N A Thorn (Institute of Medical Physiology A University of Copenhagen Denmark) STUDIES OF THE POSSIBLE RELEASE OF ANTIDIURETIC HORMONE FROM HYPOTHALAMIC TISSUE IN VITRO

It has generally been assumed that the release of neurohypophyseal hormones occurs only from the nerve endings of the supraoptic and paraventricular cells in the neurohypophysis No physiologic proof of this assumption seems to exist however It might seem possible that hormone could be released either from cell bodies or from the beginning of long axons or from endings of short axons in the hypothalamus Hormone can be extracted from the supraoptic and paraventricular nuclear regions in the hypothalamus and the cell bodies and axons contain neurosecretory granules

In the experiments to be discussed isolated pieces of infundibulum and hypothalamus kept in a medium were subjected to stimulation by potassium depolarization and various other procedures which might be suspected to cause a release In no one of the experiments were measurable amounts of vasopressin released to the medium although the tissue did contain hormone

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Boethius J and E. Knutsson (Department of Physiology Karolinska Institutet Stockholm Sweden) RESTING POTENTIALS OF DEVELOPING SKELETAL MUSCLE CELLS IN CHICK EMBRYOS

The resting membrane potentials were measured in muscle cells of the chick at various stages of development from the 3rd day *in ovo* (incubation time 21 days) until the 5th day *ex ovo* The measurements were made *in situ* with capillary micro-electrodes which were checked frequently during the experiments by impalements into frog sartorius muscle The electrodes were regarded as satisfactory only if membrane potentials of 88—91 mV were obtained In 3-day embryos the electrodes were inserted into cells of the primordial muscle tissue in the paravertebral region in later developmental stages into thigh muscle cells In each of the age groups studied measurements were made on about 100 cells penetrated at random The values within each group were widely scattered most likely due to injury of some of the cells on penetration and to varying cell types and sizes

On the 3rd day *in ovo* (about stage 22) the mean resting potential was 25 mV and the largest resting potentials were about 40 mV At this stage the primordial muscle tissue contains myoblasts with occasionally two or more nuclei and cells that cannot be distinguished from fibroblasts (Dessouky and Hibbs 1965) and no movements can be detected in the embryo During the stages from the 6th to the 15th day of incubation the mean values of the resting potentials were approximately the same but the maximal values were 60—65 mV During this period the movements of the extremities (which appear on the 5th or 6th day) successively increase in vigour and an appreciable development of the muscle cells occurs so that by the 15th day they resemble those of the adult Some time after the 15th day there is a marked increase in resting potential the mean and maximum values by the 19th day being 62 and 86 mV respectively Immediately after hatching the corresponding values were 65 and 98 mV By the 5th day *ex ovo* no further increase was observed The results so far obtained thus show that there is a rise of the resting potential of muscle fibres during the latter part of the incubation time

Dessouky D A and R G Hibbs *Amer J Anat* 1965.116.523—566

XII Scandinavian Congress of Physiology Turku 1966
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Bolme P, S H Ngai and S Rosell (Department of Pharmacology Karolinska Institutet Stockholm Sweden) INFLUENCE OF VASO CONSTRICTOR NERVE ACTIVITY ON THE CHOLINERGIC VASO DILATOR RESPONSE IN SKELETAL MUSCLE IN THE DOG

Chloralose anaesthetized and conscious dogs were used In the anaes thetized dogs the isolated gracilis muscle was perfused with blood at a constant flow rate The perfusion pressure was recorded In unana esthetized dogs blood flow was measured with an electromagnetic flowmeter probe placed around the external iliac artery The cholinergic sympathetic vasodilator nerves were activated by topical stimulation in the hypothalamus and the mesencephalon during graded levels of vaso constrictor nerve activity Increase in vasoconstrictor nerve activity was produced either by clamping one or both carotid arteries or by central topical stimulation through electrodes implanted in the vasoconstrictor areas

Activation of the cholinergic vasodilator nerves produced a vasodilator response in the skeletal muscles An increase in vasoconstrictor tone blocked the vasodilator effect when threshold stimulation intensity was used However when the vasodilator nerves were stimulated with suprathreshold intensity the vasodilator responses could then be produced even at an elevated vasoconstrictor tone The physiological significance of the experimental findings will be discussed

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Borg E. and A R Moller (Department of Physiology Karolinska Institutet Stockholm Sweden) ON THE SENSITIVITY OF THE ACOUSTIC MIDDLE EAR REFLEX TO ANESTHETICS

The acoustic middle ear reflex was studied in rabbits and humans by measuring the change in the acoustic impedance simultaneously in both ears in response to sound stimulation of either the left or the right ear The change in acoustic impedance was taken as a quantitative measure of the net activity of the middle ear muscles (Moller 1962)

Intravenous Xylocain was found to have no measurable effect on the acoustic middle ear reflex in rabbits even in doses (10—15 mg/kg) which were close to that producing convulsions In rabbits intravenous injections of 8 mg/kg Nembutal (*i e* 20 % of that required for surgical anesthesia) reduced the sensitivity of the ipsilateral reflex by 5 db and 16 mg/kg reduced the sensitivity by 10 db The contralateral reflex was influenced slightly more than the ipsilateral response

In experiments on humans 3 mg/kg Nembutal given orally reduced the sensitivity of the ipsilateral reflex by 5—7 db Experiments on the effect of alcohol were also made on humans showing that a blood concentration of 0 1 % reduced the sensitivity of the reflex by 4—6 db

Moller A Acustic reflex in man *J acoust Soc Amer* 1962.34 1524—1534

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Brundin T (Department of Physiology Karolinska Institutet Stockholm Sweden) SYNTHESIS OF CATECHOLAMINES IN PREAORTAL PARAGANGLIA AND ADRENAL GLANDS OF NEWBORN RABBITS

The incorporation and disappearance of C^{14} labelled catecholamines in preaortal paraganglia and adrenal glands have been studied in newborn rabbits after administration of labelled dihydroxyphenylalanine (DOPA-C^{14}) The organs were excised at different times after the administration of DOPA-C The catecholamines of the organs were separated on cation exchange resin columns and the radioactivity of the amines was measured by a liquid scintillation technique — Formation of noradrenaline-C^{14} could be demonstrated both in paraganglia and adrenals within 30 min after the administration of DOPA-C^{14} Adrenaline-C^{14} appeared in the adrenal glands 3 hours later The maximal adrenal content of labelled noradrenaline was found 4 hours after the DOPA-C^{14} administration while the formation of labelled adrenaline reached its peak 20 hours later In the paraganglia no adrenaline-C^{14} was found The disappearance of labelled catecholamines was slower from the paraganglia than from the adrenal glands — The functional relevance of the paraganglionic tissue will be discussed in view of the results obtained

Bugge-Asperheim B and J Kjekshus (Institute for Experimental Medical Research University of Oslo Ullevål Hospital Oslo Norway)
MYOCARDIAL FUNCTION AND OXYGEN CONSUMPTION DURING PROLONGED HYPOTENSION

The effects on myocardial function in hypovolemic and normovolemic hypotension were studied in intact Nembutal anesthetized dogs Hypovolemic hypotensive dogs were bled from an artery to a reservoir and the mean aortic pressure (AP) was maintained at 50 mm Hg for 3—4 hours

Normovolemic hypotension was obtained by a ganglionic blocking agent trimetaphan camphorate (Arfonad)

Myocardial blood flow (MF) was estimated from hydrogen desaturation curves obtained polarographically by means of a platinum electrode mounted on a cardiac catheter and placed in the coronary sinus Blood was drawn from the coronary sinus for determination of myocardial oxygen consumption (MVO_2)

Thermal indicator dilution technique was used for estimation of cardiac output (CO) and left ventricular volume

Left ventricular pressure was measured with a high frequency responding transducer which permitted estimation of the maximum rate of rise of the ventricular pulse pressure (dp/dt)

Shortly after bleeding MVO_2 was reduced to 60 % of control value but increased to 90 % during the next hours This was accompanied by an increase in dp/dt Cardiac output was reduced to 30 % during the hypotensive period and did not increase

Since the external work (CO × AP) was constant the increase in MVO_2 indicated an increase in the internal work of the heart

Normovolemic hypotension due to Arfonad reduced MVO initially to the same low level No increase was observed during the hypotensive period despite less reduction in external cardiac work No change in dp/dt was observed

The increase in dp/dt and MVO_2 reflects a prolonged stimulus during haemorrhagic hypotension

The possible roles of increased sympathetic activity endogenous catecholamine secretion and pH deviations on myocardial oxygen consumption and contractility during haemorrhagic hypotension will be discussed

This study was supported by a grant from the Norwegian Council on Cardiovascular Diseases

Carlsson B E Giacobini and S Hovmark (Department of Pharmalogy Karolinska Institutet Stockholm Sweden) AN INSTRUMENT FOR SIMULTANEOUS DETERMINATION OF Na AND K IN MICRO-SAMPLES OF BIOLOGICAL MATERIAL

In the study of the intracellular variations in Na and K in individual stretch receptor neurones of the crayfish the need arose for measurements of these ions Conventional flamephotometers could not be used because of the very small amount of ions present and the difficulty of using fluid material for analysis

Muller described the principle to measure the total emission from a biological sample placed in a gas flame We have modified his technique and improved its sensitivity

The most stable flame was obtained using a specially designed jet pressure reducing valves and flowmeters The air hydrogen flame proved most suitable The sample on the tip of a 50 μ platinum iridium wire was automatically introduced into the flame This movement started the integration storage and reading out of the signal

The emitted light was focussed through selective filters (7640Å for K 5860Å for Na) on the photomultipliers tubes The latter were maintained at optimal working temperature From these the output signals were displayed on a double beam oscilloscope and simultaneously electronically integrated The signals showed a characteristic pattern thus making possible detection of occasional improper excitation The integrated signals were fed into capacitor memories For each channel the background was subtracted by utilizing a second memory which received an integrated signal as the cooled sample free wire continued into the flame The two signals were then read out through a subtractor and registered on a potentiometric recorder

The measurements were shown to be linear in the range of 10^{-12}—10^{-11} moles This sensitivity can however be increased to measure 10^{-14} moles The simplicity and high sensitivity of the system makes it particularly suitable for determinations of ions in small samples of fluid (<10 nl) individual cells etc

Muller P *Exp Cell Res* 1958 Suppl 5 118—152

Castrén O A Pekkarinen L. Rauramo and E Soderlin (Department of Pharmacology and Department of Obstetrics and Gynecology University of Turku Finland) EFFECT OF NORMAL DAILY ACTIVITY AND HOSPITAL BED REST ON THE EXCRETION OF ESTRIOL AND PREGNANDIOL DURING NORMAL AND TOXEMIC PREGNANCY

The excretion of estriol and pregnandiol was determined simultaneously in the urine of 88 women during 24—43 weeks in non toxemic pregnancy and of 78 women during 30—42 weeks in toxemic pregnancy from the area of Turku with a low rate of toxemia of pregnancy Estriol was determined by the fluorimetric micromethod according to Pekkarinen s modification of Beling s (1963) and pregnandiol according to Pekkarinen s modification (1966) of Waldi s method (1962)

The mean pregnandiol excretion during the 10th month of nontoxemic pergnancy during hospital bed rest 37.2±2 1 mg/24 hrs was slightly but significantly lower than in the patients with non toxemic pregnancy in the Maternity Centre 45 6±2 8 mg/24 hrs (p<0 05) In toxemic pregnancy the excretion during hospital bed rest was 35 6±2.2 mg/24 hrs or about the same as in non toxemic pregnancy above However the lowest excretion was found in the patients during hospital bed rest and suffering from the severest toxemia 30 0±4.2 mg/24 hrs which excretion was not significantly lower than in the toxemic pregnant women in the Maternity Centre 42.2 ±6 7 mg/24 hrs (p>0 05)

The mean estriol excretion in the 10th month of non toxemic pregnancy during hospital bed rest 23 4±1.5 mg/24 hrs was not significantly lower than during daily activity at the Maternity Centre 26 8±2.5 mg/24 hrs Neither did the mean estriol excretion in the 10th month of toxemic pregnancy 25 6±2 1 mg/24 hrs differ from the corresponding excretion in women in normal daily activity 27 6±9 1 mg/24 hrs In the patients with severe toxemia the mean estriol excretion was only 19 0±3 6 mg/24 hrs slightly but not significantly lower than in toxemic pregnancy during normal daily activity (p>0 05)

In postmaturity the mean pregnandiol excretion decreased significantly to 26.5±3 8 mg/24 hrs (p<0 05) but not the mean estriol excretion to 18.3±2.3 mg/24 hrs

Beling C G *Acta endocr (Kbh)* 1963 Suppl 79 68
Pekkarinen A In *Research on Steroids vol II* ed C Cassano Pensiero Sciéntifico Rome 1966 233
Waldi D *Klin Wschr* 1962 40 827

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Clausen T (Institute of Physiology University of Aarhus Denmark)
CATIONS GLUCOSE METABOLISM AND INSULIN ACTION

A number of recent reports point to the fact that cations and cation transport may play a central role in the regulation of carbohydrate metabolism

This relationship was therefore investigated using isolated rat hemidiaphragms which were incubated in Krebs—Ringer bicarbonate buffer containing U^{14}C-labelled glucose

1 Active cation transport

It was shown that ouabain without affecting the glucose uptake inhibits the lactate production and stimulates the incorporation of glucose into glycogen

As essentially the same changes were obtained when K+ was omitted from the incubation medium it is suggested that they are secondary to an inhibition of the active coupled transport of Na+ and K+

Stoichiometric calculations showed that either incubation in K+ free medium or the addition of ouabain channels glucose away from glycolytic breakdown and into the glycogen pool

2 Cation milieu

When Na+ in the buffer was replaced by choline or K+ both glucose uptake lactate production and the incorporation of glucose into glycogen were diminished

When Na+ was replaced by Li+ a stimulation of glucose uptake and glucose incorporation into glycogen was produced However the lactate production was inhibited These effects of Li+ were demonstrable at concentrations down to 15 mM

Changes in cation transport and cation milieu thus seem to imitate some of the well known effects of insulin upon glucose metabolism

Furthermore it could be shown that changes in cation milieu interfere with the same insulin effects

Therefore it is discussed whether these findings could have any bearing on the mode of action of insulin

It is suggested that cations and the various conditions known to influence cation transport could be used as tools in elucidating the mechanism of insulin action upon the cell membrane

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Dahl M (Cardiorespiratory Research Unit and Department of Pediatrics University of Turku Finland) ON THE PHYSIOLOGIC MURMURS IN THE PULMONARY ARTERY REGISTERED WITH PHONOCATHETER.

41 clinically normal children from 3 months to 15 years were examined with phonocatheter The heart sounds of all were normal in auscultation and extrathoracal phonocardiography but in all a systolic murmur I°—III°/IV° was audible The maximum point was in the second intercostal space at the left sternal border Cardiac catheterization and intracardiac phonocardiography were performed in local anesthesia by taking the catheter into the heart from the right vena saphena magna During the catheterization and phonocatheterization the children were either awake or in normal sleep The pressures and oxygen content of the right heart were normal and no signs of shunt or stenosis were discovered. In twelve children it was possible to take the catheter through the foramen ovale into the left atrium In the phonocatherization the phonocatheter of AEL was used (the microphone was an activated barium titanate cylinder)

In all children a murmur was registered above the pulmonic valves The shape of the murmur was like that registered on the chest but higher in amplitude In all the murmur began with the first sound extended in the majority of the cases into the second sound but ended in part of them 0 02—0 08 sec before the second sound It was ascending in the early systole and descending in the late systole The maximum was at the end of the early systole or in the middle of the systole The murmur was in the majority of the cases about ½ of the second sound in amplitude but in some even 1¼ of the second sound The murmur decreased both in amplitude and in frequency towards the periphery of the pulmonary artery

In part of the cases with the strongest murmur it was heard against the blood flow into the middle of the right ventricle but in no case at the apex of the ventricle In these cases the murmur was also carried through the walls into the middle of the right atrium and into the superior vena cava where it was considerably fainter than in the pulmonary artery The foramen ovale with no shunt had no effect on the murmur of the pulmonary artery and on the sounds registered intra cardially

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Dahlstrom A. (Department of Histology Karolinska Institutet Stockholm Sweden) SOME EFFECTS OF RESERPINE ON THE ACCUMULATION OF NORADRENALINE IN PERIPHERAL ADRENERGIC NEURONS

The transport of NA storing granules in adrenergic neurons has been studied by means of axotomy using the histochemical fluorescence technique of Hillarp and co-workers and the biochemical spectrophotofluorimetric method It was shown that large amounts of NA rapidly accumulate above a compression of a nerve carrying adrenergic fibres and that this enormous increase in all probability not caused by local synthesis Reserpine treatment 12 and 24 h after the ligation of the nerve — which cause a disappearance of the developed accumulation — showed that the main part if not all of the accumulated NA must be localized within storage granules since the main action of reserpine is to block the storage mechanism of the granules Since the granules are complex protein structures and thus mainly or entirely synthetized in the cell body these accumulated particles must have been transported through the axon and arrested by the ligature The rate of this transport has for rat sciatic nerve been determined to 5—6 mm per hour (Dahlstrom and Haggendal 1966)

The turn-over rate of granules in the perykarya of sympathetic neurons seems to be about 4—6 hours The normally occurring NA fluorescence in adrenergic ganglion cell bodies totally vanishes after reserpine treatment and the earliest signs of a reappearance of the NA in the nerve cell bodies can be seen after 12—24 h If a ligation of the axons is performed at different times after the reserpine administration the earliest signs of NA accumulation can be seen 15—18 h after the injection of the drug thus about 30 h before the return of NA fluorescence in the nerve cell bodies In the terminals the fluorescence reappears at the earliest 3—4 days after the injection The possible explanation of this is discussed

Dahn I and P Paulev (Institute of Clinical Physiology University of Lund Sweden and Institute of Physiology University of Aarhus Denmark)
LIMB BLOOD FLOW DURING BREATH HOLDING

Measurements of the limb blood flow with a water filled venous occlusion plethysmograph were performed in three normal supine persons during rest and during breath holding The apnoea time was 120—180 sec lung volume 85—95 % of vital capacity and the airway pressure about +10 cm water The blood flow response on breath holding in air is an immediate reduction to about 20 % of the resting forearm and calf blood flow and to about 50 % of the resting hand flow After blockage of the sympathetic stellate ganglion with a local anaesthetic agent there was no reduction of the resting blood flow on breath holding The blood flow reduction was found both during breath holds in the inspiratory and the expiratory position Voluntary hyperventilation increases the resting forearm flow while apnoea decreases the forearm and calf blood flow so the decrease is most likely related to the apnoea per se

Man thus shows a reduction in limb blood flow similar to the reduction found in many other mammals during apnoea (Irving 1965) The described flow reduction is assumed to be caused by a reflex with a sympathetic efferent path

Irving L. Comparative anatomy and physiology of gas transport mechanisms In *Handbook of Physiology Respiration* Washington D C Am Physiol Soc sect 3 vol 1 chapt 5 pp 177—212 1965

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Delin N A and L W Gray, Jr* (Thoracic Surgery Research Laboratory Karolinska Institutet Stockholm Sweden) CORONARY ARTERIAL SYSTOLIC AND DIASTOLIC FLOW DURING ACUTE CONSTRICTION OF THE ASCENDING AORTA

Three dogs anesthetized with pentothal and nitrous oxide were used Flow in the ascending aorta and in the circumflex branch of the left coronary artery were measured with two electromagnetic flowmeters Pressures in the left ventricle in the aortic root and in the aortic arch were measured with strain gauge transducers

The ascending aorta was constricted to various degrees with a snare Systole was identified in the left ventricular pressure curve as the interval between the beginning of the upstroke and the beginning of the rapid phase of the downstroke

During constrictions of the ascending aorta for up to four minutes causing a systolic pressure gradient of 40 to 80 mm Hg between the aortic root and the aortic arch and a 20—40 % decrease in ascending aortic flow marked changes in coronary arterial flow pattern were seen The systolic component that is the volume of blood flowing past the measuring probe during systole increased from considerably less than half to more than half of the flow during one cardiac cycle The diastolic component decreased correspondingly Flow per time unit was on the average increased but was unchanged or even decreased in some experiments

The shift of blood flow from diastole to systole was due partly to a prolongation of systole but more to an increase of the flow level during systole

The increase in flow volume during systole represents partly a dilatation of the superficial coronary artery branches distal to the flow probe but maybe also flow through small intramyocardial vessels

*) Medical student George Washington University School of Medicine

Diamant B (Department of Pharmacology Karolinska Institutet Stockholm Sweden) CHANGES IN ATP DURING HISTAMINE RELEASE FROM RAT MAST CELLS

Anoxia inhibits histamine release from lung tissue but glucose counteracts the inhibition This effect of glucose was first observed with Ascaris extract as histamine releasing agent (Diamant 1960) and was soon confirmed in various laboratories in respect to other tissues and other histamine releasing agents The effect of glucose reflects the reactivity of the tissue mast cells Neither do mast cells undergo morphological changes nor is histamine released when the cells are exposed to histamine releasing agents under glucose free anoxic conditions Both phenomena occur if glucose is present (Diamant and Uvnas 1961 Uvnas 1964) It therefore seems that an energy requiring step is involved in the cellular reactions leading to morphological changes (degranulation) with accompanying release of histamine

Since glycolytic reactions seem to suffice as energy donator the activities of some metabolic enzymes in rat mast cells isolated from peritoneal washings were investigated by fluorometric methods devised by Lowry and Passoneau (1964) The activities were compared with those of the macrophage fraction of the same washing

Mast cells lack glucose-6-phosphate dehydrogenase 6-phosphogluconate dehydrogenase and isocitric dehydrogenase but show activities of hexokinase lactic dehydrogenase and malate dehydrogenase All these enzymes were observed in the macrophage fraction

ATP in mast cells amounts to about 2 mmoles/K dry weight When the cells had been stored at 37 C for 60 min no change was observed

When degranulation of mast cells was induced by hypotonic incubation media about 75 % of the ATP of the cells was hydrolysed during 1 hour at 37°C probably due to a concomitant release of ATPase from the mast cells On the other hand when histamine released (50 %) was induced by compound 48/80 ATP decreased only 1—18 % after 1 hour at 37 C in different experiments Similar effects were noted for lactic dehydrogenase Thus when mast cells were incubated in hypotonic media 5—6 times higher lactic dehydrogenase activity was noted in the incubation medium as compared to degranulation and histamine release induced by compound 48/80

The results show that there is a definite difference between the two ways by which the mast cells are degranulated and suggest that the degranulation process induced by compound 48/80 is a more discreet process which will not necessarily result in the loss of the metabolic integrity of the mast cell

Diamant B *Acta physiol scand* 1960.50 suppl 175 34
Diamant B and B Uvnas *Acta physiol scand* 1961.53 315—329
Lowry O H and J V Passonneau *J biol Chem.* 1964.239.31—41
Uvnas B *Ann N Y Acad Sci* 1964 *116* 880—890

Donner K. O and T Reuter (Department of Zoology University of Helsinki Finland) THE EFFECT OF METARHODOPSIN ON THE SENSITIVITY OF THE RHODOPSIN RODS IN THE FROG DURING DARK ADAPTATION

In previous work we found that the relative number of quanta absorbed necessary to produce a threshold response is proportional to the rate of regeneration of rhodopsin However the fairly rapid sensitivity increase that takes place during the first 40 min (at $+14°$) of dark adaptation cannot be accounted for in this way During this period the log threshold of the rods is linearly related to the amount of metarhodopsin which shows an exponential decay with a half return time of 4—5 min at $+15°$ This substance formed upon the bleaching of rhodopsin exists in two forms metarhodopsin I and II which are in a thermal tautomeric equilibrium that depends among other factors on temperature and pH (Matthews *et al* 1963) Experiments performed at different temperatures indicate that it is the amount of metarhodopsin II that is significant

The linear relation between metarhodopsin II and log threshold is as shown by Rushton what would be expected if the sensitivity reducing factor (here meta II) enters the feed back of a parametric feed back model as defined by Fuortes and Hodgkin to describe the potentials from cells in the ommatidia of Limulus The mode of action of metarhodopsin II remains obscure but it is interesting to note that the bleaching of rhodopsin causes a drop in the internal equivalent pH of the rods with a corresponding shift of the metarhodopsin equilibrium towards metarhodopsin II

In frog dark adaptation we thus have two effects that reduce rod sensitivity 1 the effect of bleaching mediated by metarhodopsin II and 2 the effect of the rate of regeneration

Matthews R G R Hubbard P K Brown and G Wald Tautomeric forms of metarhodopsin *J gen Physiol* 1963 47 215—240

Edman K. A P and A Kiessling (Department of Pharmacology University of Lund Lund Sweden) THE TIME COURSE OF THE ACTIVE STATE OF THE FROG SKELETAL MUSCLE FIBRE IN RELATION TO THE SARCOMERE LENGTH

Up to the present time there has been no clear demonstration as to whether or not the time course of the mechanical activity of the skeletal muscle cell is related to the length state of the contractile system The present experiments have been designed to investigate this point by recording the active state course in isolated muscle fibres (semitendinosus muscle of the frog 2—3 C) at various degrees of extension of the sarcomere pattern

The techniques used for recording of tension and changes of the striation spacing during activity have been described previously (Edman 1966) The fibres were paced to produce a sequence of four partially summated twitches (frequency 4—5 c.p.s.) at a pre set length at intervals of 90 sec The decay phase of the active state was determined by means of quick release in the fourth cycle the amount of release being carefully controlled (0 1 μ per sarcomere spacing) The rising phase of the active state curve was defined as described previously (Edman Grieve and Nilsson 1966) The maximal intensity of the active state was given by the tetanic output at the length considered

Reduction in length of the contractile system caused a graded decrease in the duration of the active state In 6 fibres investigated the duration of the active state at 1 9 μ sarcomere length was 48—84 (mean 71) percent of the active state duration existing at 2 6 μ as measured at 50 per cent of maximal activity Changing from one length to another did not have any substantial effect on the membrane action potential as determined by intracellular recording using a standard glass electrode or at measurements below slack length (2.3—1 8 μ) by means of a movable Brady Woodbury type of glass electrode

It is concluded that 1 the degree of extension of the muscle cell is a determinant of the time course of the mechanical activity although the dependence on sarcomere length varies quantitatively from fibre to fibre 2 the effect on the active state course produced by a length change is not to be attributed to a change in the duration of the action potential (see Edman Grieve and Nilsson 1966) the degree of extension of the cell probably influences a more intimate step in the excitation-contraction process

Edman K. A P., *J Physiol (Lond.)* 1966.183 407
Edman K A P D W Grieve and E Nilsson *Pflüg Arch ges Physiol* 1966 In press

XII Scandinavian Congress of Physiology Turku 1966
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Ekblom B (Department of Physiology Gymnastiska Centralinstitutet Stockholm Sweden) THE EFFECT OF TRAINING ON CIRCULATION DURING PROLONGED SEVERE EXERCISE

The circulatory response to prolonged severe exercise has been studied on 8 subjects before and after 4 month s hard physical training Oxygen uptake pulmonary ventilation heart rate cardiac output with the dye dilution technique stroke volume intra arterial blood pressure and blood lactate concentration were measured during submaximal and maximal exercise on a bicycle ergometer The results will be discussed

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Ekman A., V Manninen J Rastas and S Salminen (Wihuri Research Institute and Institute of Technology Helsinki Finland) THE DISTRIBUTION OF ALKALI IONS IN RED CELLS

The simultaneous movements of four alkali ion tracers ^{22}Na ^{42}K ^{86}Rb and ^{137}Cs were followed in high K (man) and low K (cat) red cells at 38°C and 6 C *The movement of non radioactive Li was examined separately* From the inflow and outflow data the stationary distribution ratio R_∞ of the ion concentration of the medium to that of the cell was calculated For human red cells at 38 C the values of R_∞ were Li 1.8 ^{22}Na 2.3 ^{42}K 0.10 ^{86}Rb 0.08 and ^{137}Cs 0.33 The half exchange times in the same experiments were Li 6.0 h ^{22}Na 1.5 h ^{42}K 35 h ^{86}Rb 38.5 h and ^{137}Cs 45 h. The low K (cat) red cells showed little preference either for ^{42}K or *for ^{86}Rb or ^{137}Cs over ^{22}Na In the cold the tracer movements were similar* in human and cat red cells The differences in the behaviour of the alkali ions in the high K cells at 38 C can be considered to be related to their different mobilities

Elwin C E. and S Andersson (Department of Pharmacology Karolinska Institutet Stockholm Sweden) INFLUENCE OF pH ON RELEASE OF GASTRIN BY CHEMICAL AGENTS

A variety of compounds such as acetylcholine choline amino acids and alcohols can cause release of gastrin when applied on the antral mucosa The release is blocked by atropine suggesting a cholinergic mechanism

Gastrin release has previously been shown to be inhibited by a low pH of the antrum The mechanism of the inhibitory effect of high acidity is unknown In the present experiments the inhibitory effect of different pH levels in the antrum was studied on gastrin release produced by acetyl choline choline glycine and ethanol

Dogs were prepared with isolated fundic and antral pouches The antral pouches were continuously perfused at a constant rate with graded con centrations of the test solutions each test sample being perfused during $1\frac{1}{2}$ hours pH of the solutions was adjusted by buffers except at pH 1 0 when pure 0 1 M hydrochloric acid was used. Each substance was tested at pH 1 3 5 7 and 9 The response following perfusions of the antral pouches was determined as the secretory output of acid from the fundic pouches

The pH-dependency of gastrin release varied with the substances tested. To inhibit ethanol stimulated gastrin release a pH of 1 0—1 5 was neces sary whereas with glycine inhibition occurred at pH 3 0—5 0 The sen sitivity to acidity of gastrin release caused by acetylcholine and choline was similar to that of glycine With lower concentrations of choline (1—2 %) pronounced inhibition was observed at pH 5 0 whereas no inhibition occurred with the highest concentration (8 %) To inhibit completely the response to 8 % choline solution the pH had to be reduced to 1 0

The significance of these findings in regard to the mechanism where by acidity inhibits release of gastrin will be discussed

Engberg I A Lundberg and R W Ryall (Department of Physiology University of Goteborg Sweden) THE EFFECT OF RESERPINE ON TRANSMISSION FROM THE FLEXOR REFLEX AFFERENTS

The monoaminergic nerve terminals in the spinal cord belong to descending pathways (Carlsson *et al* 1964) Since the monoamine precursors L-3 4-dihydroxyphenylalanine (DOPA) and 5-hydroxytryptophane depress transmission from the flexor reflex afferents in the spinal cord there is the possibility that NA (noradrenaline) and 5 HT (5-hydroxytryptamin) may be transmittors from descending pathways with this function (Anden *et al* 1964) A further test is now provided by analysis of the effect of reserpine which liberates the transmitter from monoaminergic terminals

After administration of Nialamide to inhibit the monoamine oxidase an intravenous injection of reserpine (2—3 mg/kg) effectively depresses transmission from the FRA to primary afferents and motoneurones in acute spinal cats In chronic spinal cats (two weeks after transection of the spinal cord in the lower thoracic region) reserpine after Nialamide has no effect on transmission from the FRA A partial reversal of the reserpine effect in the acute spinal cat is obtained by an intravenous inject on of the 5-HT antagonist Deseril (3 mg/kg) but the adrenergic α receptor blocker phenoxybenzamine (20 mg/kg) has no corresponding effect It is postulated that reserpine at least partly acts through liberation of 5-HT from a descending 5-hydroxytryptaminergic pathway No conclusion can be drawn regarding whether reserpine also acts through liberation of NA

Andén N E M G M Jukes and A Lundberg *Nature (Lond)* 1964.202 1222—1223
Carlsson A B Falck K Fuxe and N A Hillarp *Acta physiol scand* 1964 *60* 112—119

Ferngren H, A Brock A Elmqvist G Eneroth and H Rydin (Department of Biology National Pharmaceutical Laboratory Stockholm Sweden)
SOME EXPERIENCES IN EVALUATING TESTS FOR THE TERATOGENIC ACTION OF DRUGS WHICH ARE REGISTERED OR UNDER APPLICATION FOR REGISTRATION IN SWEDEN

The last few years there has been an explosive increase in experimental data concerning teratogenic effects of old and new drugs As many views exist on how such experiments should be carried out the difficulties in the evaluation are considerable

At the National Pharmaceutical Laboratory from 1963 to the end of 1965 we have evaluated data from the manufacturers on teratogenic tests for 91 different drugs The following parameters have been found valuable for the statistical comparison of test and control groups number of malformed animals number of resorptions number of stillborns and number of young surviving the neonatal period 29 of the 91 evaluated drugs showed a statistically significant deviation in the Chi square test ($P<0.05$) in one or more of these parameters (only 5 concerned malformations) 24 of the 29 positive drugs had been tested in more than one species and of those 4 showed statistically significant deviations from controls in two species Of great importance for the evaluation is the magnitude of the dose causing the significant effects and its relationship to the toxic dose for females

Flemstrom G and B Frenning (Institute of Physiology and Medical Biophysics University of Uppsala Sweden) **DIFFUSION OF ACETIC ACID AND DURATION OF THE PERMEABILITY EFFECTS ON THE GASTRIC MUCOSA**

The permeability of the gastric mucosa is greater for weak acids than for hydrochloric acid It has been suggested that this is due to the ability of non-dissociated weak acids to pass through the lipid parts of the cell membrane (5 4)

In order to study the diffusion mechanism [14]C-labelled acetic acid was instilled into non secreting cat stomachs isolated by ligatures from the oesophagus and duodenum The volume of the intragastric contents was measured every fifteen minutes samples being taken for analysis of hydrogen sodium potassium acetate and chloride ions

The decrease in concentration after 60 minutes was greater for hydrogen ions than for acetate ($P<0.05$) while the increase in concentration of sodium and potassium was greater than that for chloride ($P<0.02$) This suggests that in addition to acetic acid escape there is a small degree of exchange between hydrogen and sodium potassium ions

Acetic acid has been shown to have an inhibitory effect on gastric *secretion which lasts at least 24 hours (Babkin 1950) It has also been* reported that acetic acid increases the permeability of the gastric mucosa to various ions (Davenport 1964 Flemstrom *et al* 1964) The duration of this permeability increase has been studied by making five consecutive instillations on the same cat of a/HCl b/HAc c/HCl d/HCl and e/HCl The mean permeability coefficients were a/0 11±0 01 b/0.25±0 02 c/0.22 ±0 01 d/0 17±0 01 and e/0 13±0 01 ml/min The permeability thus returned to almost normal after 90 minutes and therefore a permeability increase alone cannot explain the inhibitory effect of acetic acid on the secretory mechanism for gastric juice

Babkin B P *Secretory mechanism of the digestive glands* 2nd ed New York 1950
Davenport H W *Gastroenterology* 1964 46.245—253
Flemstrom G B Frenning and K J Öbrink *Acta physiol scand.* 1964 62 422—428
Schanker L S P A Shore B B Brodie and C A M Hogben *J Pharmacol exp Ther* 1957.120 528—539
Teorell T *J gen Physiol* 1939.23 263—274

Folkow, B B Lisander and S C Wang (Department of Physiology University of Goteborg Goteborg Sweden) CHANGES IN CARDIAC OUTPUT IN CATS DURING STIMULATION OF THE HYPOTHALAMIC DEFENCE AREA AND THE BULBAR DEPRESSOR AREA

Defence area stimulations induce a characteristic sympathetic pattern involving cholinergic muscle vasodilatation increased flow resistance in most other tissues and increased arterial pressure and heart activity (cf Uvnas 1960) — Stimulations of the bulbar depressor area relaying most central and reflex sympatho-inhibitory responses (cf Lofving 1961) also produce an initial muscle blood flow increase However this is caused by inhibition of sympathetic tone reducing flow resistance in other tissues as well as arterial pressure and heart activity

Cardiac output (CO) was determined with the thermodilution method in anesthetized-curarized cats where arterial and central venous pressures heart rate and muscle blood flow were also recorded

During control CO was about 500 ml/min (body weight 3 kg) Defence area stimulations producing up to five fold increases in muscle blood flow promptly increased CO up to 100 per cent and stroke volume and arterial pressure 50—60 per cent There was however little change of central venous pressure — Atropine block of the cholinergic muscle vasodilatation greatly enhanced the arterial pressure rise and correspondingly reduced the CO increase with the result that the increased work load for the heart during stimulation was roughly the same as before atropine — If instead the adrenergic beta receptors were blocked (Hassle 56/28) the CO increase was largely abolished and the arterial pressure rise reduced with little change of the initial muscle vasodilatation However central venous pressure now rose revealing the venous capacitance constriction which was formerly balanced by the powerful sympathetic drive on the heart

Depressor area stimulations produced initially a slight CO increase concomitantly with the transient muscle flow increase and fall in arterial pressure However within 10—15 seconds this CO increase was changed to a CO reduction being especially marked if hydrostatic factors had increased the demand for reflex capacitance adjustments during the control phase Block of cholinergic or beta adrenergic receptors hardly affected the level of this stimulation induced CO reduction

Lofving B *Acta physiol scand* 1961 53 Suppl 184 1—82
Uvnas B *Handbook of Physiology Neurophysiology* Washington D C Am Physiol Soc 1960 sect I vol II chapt 44 pp 1131—1162

XII Scandinavian Congress of Physiology Turku 1964
Acta physiol scand 1966.68 Suppl 277

Folkow B N J Nilsson and L. R. Yonce (Department of Physiology and Department of Clinical Physiology University of Goteborg Sweden)
EFFECTS OF DIVING ON CARDIAC OUTPUT IN DUCKS

The paucity of data on the cardiac output (C O) of diving animals prompted the present investigations in unanaesthetized ducks weighing around 3 kg C O was measured by the cardiogreen and/or thermodilution methods Mean resting C O was 1495 ml/min (S D.±245) and mean heart rate 239 beats/min (S D ±78)

After one minute of submersion C O had generally decreased below 150 ml/min and heart rate below 30 beats/min The more 'habituated the duck was to the enforced submersion the more intense the bradycardia was during the dive In one case C O was reduced to 66 ml/min and heart rate to 14/min with little change of arterial pressure Stroke volume tended to decrease during the dive despite an increase of central venous pressure of 10—20 mm Hg suggesting a negative inotropic effect on the ventricles This negative inotropic effect was shown to be of vagal origin as well as influenced by an inhibited sympathetic discharge to the heart

Skeletal muscle blood flow virtually stopped during the dive while blood flow in the web where hardly any oxygen was extracted was almost unchanged This suggests that in the presence of an intense generalized vasoconstriction the A V anastomoses in apical skin sections may provide the shunt necessary to move the oxygen containing venous blood towards the heart

During the immediate 1—2 sec postdive period the heart rate increased *to 300—500 beats/min with a concomitant rise in arterial* pressure Within 10—20 seconds a profound vasodilatation appeared with central venous and arterial pressures returning towards normal C O was now of the order of 3—4 000 ml/min up to 50 times that present at the peak of the dive response only 15—20 seconds earlier Stroke volume was raised above the *resting level Then C O returned gradually to normal in 5—6* minutes Most of the postdive rise in C O was abolished by β receptor blocking agents revealing the intense sympathetic drive on the heart during this period

Frankenhaeuser B and J Lannergren (Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden) THE EFFECT OF CALCIUM ON SINGLE TWITCH MUSCLE FIBRES IN XENOPUS LAEVIS

The effect of external calcium on the potassium contractures has been reinvestigated (cf Luttgau 1963) Isolated twitch muscle fibres from the iliofibularis muscle were mounted to a strain gauge and arrangements were made to allow rapid changes of the external solution A change of [Ca] caused a shift of the S-shaped curve relating tension to $[K]_o$ A five fold increase in $[Ca]_o$ was equivalent to a one and a half fold increase in $[K]_o$ The [Ca] also affected the curve relating the contractile activity available in the fibre to the $[K]_o$ Available contractile activity was measured by applying high $[K]_o$ The change in $[Ca]_o$ acts rapidly and the described effects seem to be mediated through the membrane of the muscle fibre

Luttgau H C *J Physiol (Lond)* 1963.168 679—697

XII Scandinavian Congress of Physiology Turku 1966
Acta physiol scand 1966 68 Suppl 277

Fredholm B and Ö Haegermark (Department of Pharmacology Karolinska Institutet Stockholm Sweden) HISTAMINE RELEASE FROM MAST CELL GRANULES INDUCED BY BEE VENOM

It has been shown that bee venom degranulates mast cells by a mechanism similar to that of compound 48/80 and antigen This effect was originally assumed to be due to the phosphatidase A activity of the venom Later however the phosphatidase A activity was separated from the mast cell degranulating activity

We had observed that bee venom also releases histamine from isolated mast cell granules in ion free solution while compound 48/80 lacks this ability Thus it was considered to be of interest to study the effect of fractionated bee venom on mast cell granules

The granules were obtained by sonic disintegration of rat peritoneal mast cells and subsequent differential centrifugation in ion free sucrose solution Bee venom was fractionated by the gel filtration technique Phosphatidase A activity was assayed by the hydrolytic splitting of lecithin

We found that the phosphatidase A containing fraction — which has no effect on intact mast cells — induced histamine release from the mast cell granules while the mast cell active fraction did not In an attempt to ascertain whether the histamine release was due to the phosphatidase A content of the former fraction the processes of histamine release and phosphatide hydrolysis were compared The influence of temperature pH and enzyme inhibitors on these two reactions was investigated The reactions were found to run parallel The mechanism of histamine release from the granules will be discussed

Fredholm B and O Haegermark (Department of Pharmacology Karolinska Institutet Stockholm Sweden) HISTAMINE RELEASING FACTORS IN BEE VENOM

Bee venom is known to contain high amounts of phosphatidase A It has been shown to release histamine from various tissues although the release mechanism is not the same in different animals The histamine releasing capacity was formerly thought to be a consequence of the phosphatidase content of the venom However it is now possible to separate bee venom into several fractions and it has been shown that in different species the activity is due to different fractions (Fredholm and Strandberg 1966 Fredholm and Westerholm 1966)

The histamine release from rat peritoneal mast cells has been studied in the present work Bee venom was separated into three fractions by gel filtration The one containing phosphatidase A activity had no effect on mast cells The histamine releasing activity was found in two subsequent fractions The influence of pH temperature and enzyme inhibitors was studied as well as the time course of the reaction The results suggest that one of the factors (Fraction II) releases histamine by a mechanism similar to that of the synthetic histamine liberator Compound 48/80 and of antigen i e by activating an energy dependent enzymatic reaction in the cell The other factor (Fraction III) which is a direct lytic substance has a different more unspecific mode of releasing histamine

Fredholm B and K Strandberg 1966 To be published
Fredholm B and B Westerholm 1966 To be published

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Fuxe K and T Hokfelt (Department of Histology Karolinska Institutet Stockholm Sweden) EXPERIMENTALLY AND PHARMACOLOGICALLY INDUCED CHANGES IN THE AMINE LEVELS OF CENTRAL MONOAMINE NEURONS AS STUDIED WITH A HISTOCHEMICAL TECHNIQUE

With the histochemical fluorescence technique for the cellular demonstration of dopamine (DA) noradrenaline (NA) and 5-hydroxytryptamine (5-HT) it has been possible to demonstrate the existence of central monoamine neurons Several systems have been mapped out The following pharmacological experiments have been made 1 Reserpine Functional recovery after reserpine seems to be correlated with the ability of the amine granules to take up amines 2 Segontin This drug seems to be a shortacting blocker of the amine storage function of the granules and depletes the neurons of their amines in the same way as tetrabenazine 3 Haloperidol After treatment with this drug certain CA cell body groups show a marked increase in their amine levels This may be due to an increased granule formation induced by the functional blockade caused by haloperidol The same phenomenon is observed after reserpine 4 Desmethylimipramine This drug seems to block the amine concentrating mechanism at the level of nerve cell membrane in the central NA but not DA neurons 5 Amphetamine This drug seems to release extragranular amines from DA and NA neurons even in low concentrations The neocortical NA nerve terminals are particularly sensitive to this drug 6 Diethyldithiocarbamate This drug causes a marked and selective depletion of the amine content of brain NA nerve terminals 7 Various drugs have been found to induce changes in the turnover rates of various central CA terminal systems as revealed by potent inhibitors of the first rate limiting step in the CA synthesis

Fyhn H J (Institute of Zoophysiology University of Oslo Blindern Norway) FUNCTION OF THE ISOSMOTIC INTRACELLULAR REGULATION DURING MOULT IN THE SHRIMP CRANGON VULGARIS

The act of shedding the old exoskeleton in Crustacea is named ecdysis It s mechanism is partly known It involves an abrupt increase in the osmolarity of the heamolymph and almost concomitantly the water content of the body increases due to osmosis leaving the body in a swollen condition The osmotic intake of water has also the effect of diluting the heamolymph and the original osmolarity is re-established soon after ecdysis Supposedly therefore the system of the extracellular osmotic regulation is activated during ecdysis The question arises if also the system of the isosmotic intracellular regulation (Duchâteau and Florkin 1956) is affected

In order to elucidate this question experiments have been made on the shrimp *Crangon vulgaris* The methods applied in the present study have previously been described in papers from this laboratory (Lange 1964) and the preliminary results may be summarized as follows

In agreement with the general findings in Crustacea it was found that the osmolarity of the shrimp s heamolymph suddenly increased just prior to ecdysis This event was rapidly followed by a water absorbtion which decreased the osmolarity of the heamolymph Furthermore it was found that the water content of the tissues increased and also that the intracellular concentration of free ninhydrin positive substances varied in parallel to the osmolarity of the heamolymph These findings indicate that also the intracellular osmolarity is regulated during ecdysis At present however it is difficult to decide whether the intra or the extracellular system of osmotic regulation is the initiating system The data seem though to support the hypothesis that the isosmotic intracellular regulation takes active part in ecdysis

Duchateau Gh and M Florkin 1956 *J Physiol (Paris)* 8 520
Lange R *Comp Biochem Physiol* 1964.13 205—216

Fyro B (Department of Pharmacology Karolinska Institutet Stockholm Sweden) REDUCTION OF ANTRAL GASTRIN ACTIVITY PRODUCED BY ELECTRICAL VAGAL STIMULATION

Release of gastrin from the antral mucosa of the stomach has previously been demonstrated only indirectly by means of acid secretion from indicator pouches A technique developed in our laboratory for assaying the secretory activity of gastrin preparations on non anesthetized gastric fistula cats (with histamine as the reference standard) (Uvnas and Emås 1961) offered the possibility to determine quantitatively the antral gastrin activity

It is known that vagal impulses stimulate gastric acid secretion partly by releasing gastrin In the present study the gastrin activity in antral mucosa of cats was determined after graded electrical stimulations of the vagal nerves Stimulation for 2 and 4 hours at 5 10 and 20 impulses/sec produced a decrease of 50—70 % of the gastrin activity Thus electrical vagal stimulation can appreciably diminish the antral gastrin content A similar decrease of the gastrin content in the antral mucosa may well occur under physiological vagal activation This assumption is supported by the finding that the antral mucosa contains only 50 % of its normal gastrin activity 3 hours after feeding a meal

Uvnas B and S Emås A method for biological assay of gastrin. *Gastroenterology* 1961 40 644

Gidlof A (Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden) INTRACELLULAR ASPECTS OF RELEASE PHENOMENA IN α EXTENSOR MOTONEURONES OF THE CAT

Release of excitation in alpha motoneurones can be obtained by removal of tonic inhibition emanating e g from the cerebellum or from a portion of the spinal cord below the one investigated (the Schiff Sherrington phenomenon) The intracellular aspects of such a removal of inhibition have been studied during reversibly and non traumatically elicited states of release in forelimb extensors of decerebrate cats To this end a technique of selective cooling has been employed

It has been shown that the membrane of the alpha motoneurones depolarises 10—15 mV during release Various other parameters undergo concommitant changes

The ascending tonic inhibition is not affected by complete deafferent ation of the lumbar spinal cord enlargement and earlier findings (Ruch 1936) to the effect that the tonic ascending inhibition revealed by the Schiff Sherrington phenomenon is not originated in the periphery of the hindlimb are confirmed

Ruch T C *Amer J Physiol* 1936 *114* 457—467

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Acta physiol scand 1966 68 Suppl 277

Green D G and J-O Kellerth (Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden) INTRACELLULAR AUTOGENETIC EFFECTS OF MUSCULAR CONTRACTION ON FLEXOR MOTONEURONES

Intracellular recordings have been made from cat spinal motoneurones innervating the ankle flexor muscles of the left hindlimb Except for the muscle nerves of the ipsilateral tibialis anterior and extensor digitorum longus muscles both hind limbs were completely denervated These two muscles could be made to contract by electrical stimulation of the peripheral stumps of the cut ventral roots

The autogenetic intracellular effects produced by flexor muscle contraction were studied during various muscle extensions and stimulus strengths to the ventral root The results thus obtained were found to differ markedly from those obtained in a similar series of experiments dealing with intracellular autogenetic effects of extensor muscle contraction (Granit Kellerth and Szumski 1966)

During contraction of the slack flexor muscles i e when no load was applied to the muscles an autogenetic postsynaptic inhibition was regularly seen to be produced indicating inhibitory end-organs with a high tension sensitivity However when applying an initial tension to the muscle this inhibition which had its peak slightly after the peak of the contraction was shortened in duration This diminution of the inhibition was caused by excitatory activity appearing on both sides of the inhibition *i e* during the rising and the falling phase of the contraction With increasing extension or stimulus strength this early and late excitation occasionally became *powerful enough completely to overrule the inhibition* which often did not increase markedly with increasing tension. These results were obtained at stimulus strengths to the ventral roots considered not be be adequate to activate the small γ-motor fibres

Interpretations of these findings will be given in terms of responses of the peripheral muscle receptors and the innervation of the latter

Granit R. J-O Kellerth and A J Szumski Intracellular autogenetic effects of muscular contraction on extensor motoneurones The silent period *J Physiol (Lond.)* 1966 182 484—503

Grillner S T Hongo and S Lund (Department of Physiology University of Goteborg Sweden) DESCENDING PATHWAYS WITH MONOSYNAPTIC ACTION ON MOTONEURONES

Monosynaptic excitatory postsynaptic potentials (EPSPs), can be recorded in motoneurones of the lumbosacral cord when the thoracic cord is stimulated (Eide Lundberg and Voorhoeve 1961) The supraspinal origin for these actions has been investigated The lateral vestibular nucleus (Deiters) is the dominating source for the action to extensor motoneurones (Lund and Pompeiano 1965) whereas the corresponding effects to flexor motoneurones can be evoked only from a more medially located region in the brain stem probably the reticular formation (Grillner and Lund 1966) However stimulation of the medial region may evoke monosynaptic EPSPs in motoneurones to some extensor muscles The pathway from the medial region is situated close to the vestibulospinal pathway in the ventral quadrant A detailed description of the contribution of monosynaptic EPSPs from the two supraspinal centres to different motor nuclei of the lumbosacral cord will be given

Eide E A Lundberg and P Voorhoeve *Acta physiol scand* 1961.53 185
Lund S and O Pompeiano *Experientia (Basel)* 1965.21 602
Grillner S and S Lund 1966 In press

XII Scandinavian Congress of Physiology Turku 1966
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Grillner S., T Hongo and S Lund (Department of Physiology University of Goteborg Sweden) INTERACTION BETWEEN THE INHIBITORY PATHWAYS FROM THE DEITERS NUCLEUS AND IA AFFERENTS TO FLEXOR MOTONEURONES

Thus investigation concerns the inhibitory actions from the Deiters nucleus in spinal motoneurones Monosynaptic excitation of extensor motoneurones from this area has earlier been reported (Lund and Pompeiano 1965)

Intracellular recording was made from antidromically identified motoneurones in lumbosacral cord A tungsten electrode was placed in the Deiters nucleus which was identified by extracellular recording of the field potential evoked by an antidromic volley in the vestibulospinal tract A transsection was made in the lower thoracic cord leaving only the ipsilateral ventral quadrant intact

Stimulation of Deiters nucleus gives a short latency IPSP in flexor motoneurones The segmental latency of this IPSP is 1.3 msec suggesting a disynaptic linkage In the knee flexor motoneurones it was regularly found that transmission of a submaximal Ia IPSP from quadriceps could be facilitated by stimulation of Deiters nucleus at a strength that was subthreshold for evoking effect in the motoneurone under investigation The time course of this facilitation suggests that volleys in the vestibulospinal tract evoke a monosynaptic EPSP in the interneurones transmitting the reciprocal Ia IPSP to those flexor motoneurones There was on the other hand neither facilitation from Deiters nucleus of the reciprocal Ia inhibitory pathway from flexor to extensor motoneurones nor facilitation of the pathways from Ib afferents and the flexor reflex afferents to flexor and extensor motoneurones

Previous investigations have shown that the corticospinal and the rubrospinal tracts give excitatory action to interneurones of spinal reflex pathways Hence the vestibulospinal tract represents a third pathway with such an effect However the finding that only one specific reflex path is facilitated from the vestibulospinal tract may imply that the interneurones of the reciprocal Ia inhibitory path are utilized to distribute inhibition also from the vestibulospinal tract

Lund S and O Pompeiano *Experientia (Basel)* 1965.21 62

Grangsjo G (*Institute of Physiology and Medical Biophysics University of Uppsala Uppsala Sweden*) EFFERENT IMPULSES IN THE RENAL NERVES

The kidney is richly supplied with sympathetic nerve fibres derived from the celiac and the superior mesenteric ganglia but there are also direct branches to the renal plexus from the adjacent portion of the sympathetic trunk There is some controversy concerning the para sympathetic supply to the kidney which most authors believe is non existant The efferent sympathetic fibres not only follow the renal arteries to terminations in the smooth muscle of the afferent and efferent vessels of the glomeruli but also terminate between the tubular cells especially in the proximal region The role of the vasomotor innervation to the kidney is certainly largely if not entirely vasoconstrictor but the function of the parenchymal innervation is rather uncertain The impulse pattern in the renal nerves was studied on chloraloseanesthetized cats Considerable difficulties were encountered in obtaining satisfactory recordings and many different shapes of electrodes were tested A very small platinum electrode was found most useful and when inserted in the nerve gave action potentials of acceptable magnitude The aortic pulse curve ECG and breathing frequency were recorded simultaneously In similarity to the results of other authors impulse showers correlated to the pulse curve were recorded Recordings were taken under normal (apart from anesthesia + exposure of kidney) conditions and during pathological states such as respiratory acidosis and hypovolemic chock i e conditions when the blood supply to the kidney is greatly diminished

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Grangsjo G H R. Ulfendahl and M Wolgast (Institute of Physiology and Medical Biophysics University of Uppsala Sweden) CONCENTRATING ABILITY OF A COUNTER CURRENT MODEL OF THE VASA RECTA SYSTEM

It is generally accepted that a counter current system in the renal medulla is responsible for the urine concentration Two counter current systems exist in the medulla namely the urine system — Henle s loop — and the blood system — the vasa recta The blood in the vasa recta has to supply nutriment to the medullary tissue but it may also take part in the concentrating mechanism According to the counter current hypothesis (Hargitay and Kuhn 1951) there must be a driving force (cf Hargitay and Kuhn s Einzeleffekt) for the system Roskenbleck and Niesel (1963) suggested that the addition of an osmotically active substance to the blood in the inner part of the medulla might give a concentrating effect and this hypothesis was confirmed in model experiments

There is another possible effect of the medullary blood flow A low O_2-content — depending on the O-shunt in the outer medulla — and a high CO_2-content in the papillary region increase the amount of water in the red cells according to the chloride shift This water drag might act as an Einzeleffekt and the result might be that the vasa recta function as a counter current multiplier system In a model consisting of a narrow 'U -channel the limbs of which were separated by a cellophane membrane oxygenated blood was slowly perfused thus passing the two sides of the membrane in opposite directions At the bend of the 'U the blood was passed through a PVC-tube in a CO_2-atmosphere and the red cells were partly deprived of their O_2 and CO was added The electrolyte concentration at the tip was found to increase during perfusion

Hargitay B and W Kuhn Das multiplikations prinzip als Grundlage der der Harnkonzentrierung in der Niere *Zeitschr Elektroch u Physikal chem* 1951.55.539—558

Roskenbleck H and W Niesel Gekoppelte Gegenstromsysteme als Modell der konzentrierenden Niere *Pflugers Arch ges Physiol* 1963.277.316—324

Gunne L-M and T Lewander (Department of Physiology Karolinska Institutet Stockholm Sweden) MONOAMINE CHANGES IN RAT BRAIN AFTER LONG TERM TREATMENT WITH DEPENDENCE PRODUCING DRUGS

Brain monoamine levels were determined after acute and chronic treatment with amphetamine and phenmetralin (Preludin®) In acute experiments dl amphetamine (20 mg/kg i p) was shown to decrease brain NA level while DA was increased 3 hrs after injection After chronic treatment (20 mg/kg i p twice daily for 6 days) both amines decreased
Phenmetralin had no effects on monoamine levels in acute experiments (40 mg/kg i p) and caused a slight increase when chronically administered (20 mg/kg twice daily for 6 days last dose 40 mg/kg) — The urinary excretion of NA and A has been found to increase 3- to 5 fold during dl amphetamine treatment (32 mg/kg i p daily) and 8- to 10-fold during phenmetraline treatment (40 mg/kg i p daily) Lewander 1966)

Lewander T Urinary excretion of catecholamines during long term administration of amphetamine and phenmetralin 1966 To be published

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Haapanen L. and C R. Skoglund (Department of Physiology Karolinska Institutet Stockholm Sweden) APPLICATION OF HIGH FREQUENCY REFLECTOMETRY FOR DYNAMIC RECORDING OF THE IONIC EFFLUX DURING ACTIVATION OF NITELLOPSIS OBTUSA

Conventional methods of studying the ionic fluxes in excitable tissue e g by tracers allow chemical identification of the ions involved but have a poor time resolving power as compared with the potential recording and besides the sensitivity of the methods used so far has been too low to permit quantitative determinations of the fluxes during a single activation process

A first series of experiments on internodal cells of *Nitellopsis obtusa* (Haapanen and Skoglund 1963) demonstrated that it is possible to record the ionic outflow during excitation by measuring the conductance changes in the extracellular medium and this method has now been further developed By means of a specially designed probe the high frequency field from an oscillator (1085 kc/s) is applied to a small volume element of the fluid surrounding the algae The increase in ionic concentration associated with the passage of an impulse is reflected to the oscillator as a variation in the resistive loading exerted on the resonant input circuit (ΔIg) and also as apparent changes in probe capacitance (ΔC) Either effect — in the range of conductivities up to 5 x 10^{-5} ohm^{-1} cm^{-1} preferably the ΔIg — is displayed simultaneously with the action potential on an oscilloscope or an inkwriter the long term stability allowing a quantitative monitoring of the changes in ionic concentration occurring during hour long experiments Application of the principle of measuring the phase shift between two loosely synchronized high frequency oscillators (Haapanen 1962) makes the instrument (HF reflectometer) sufficiently sensitive to allow ΔC-recording of the increase in ionic concentration accompanying a single action potential even at the relatively high conductivity 2 4 x 10^{-4} ohm^{-1} cm^{-1} of the natural lake water of the algae

Subsequent chemical analysis by conventional methods showed that the ions involved in the process studied are Cl^- and K^+ in approximately the same amounts averaging from 2—4x10 pmole/cm impulse

Haapanen L. *Electronic Engng* 1962.34 183
Haapanen L and C R Skoglund *Acta physiol scand* 1963.59.297

Hakulinen A (Department of Pharmacology and Department of Pediatrics University of Turku Finland) ON THE EXCRETION OF VANILMANDELIC ACID (VMA) IN DIFFERENT AGE GROUPS OF CHILDREN AND IN CERTAIN CLINICAL CONDITIONS

The VMA excretion of the newborn increases on the first few days after birth The mean VMA excretion (μg/kg) of 16—30 fullterm infants was 35 μg 54 μg 75 μg and 84 μg on 1—2 and 4—5 consecutive days and that of 7—15 premature infants 40 μg 61 μg 74 μg and 86 μg on 2—5 consecutive days after the birth Thus no significant difference per kg of body weight in the VMA excretion was seen in these two groups The prolonged delivery or the Caesarean section did not increase the VMA excretion

In the day nursery children (42 children of 2—4 years) the mean excretion of VMA was 1 61 mg/24 hrs and 118 μg/kg/24 hrs in kindergarten children (19 children of 4—6 y) 1 93 mg and 103 μg in children of kindergarten and children s summer camp (24 children of 6—8 y) 2.35 mg and 98 μg and in boys of juvenile reform school (26 boys of 12—16 years) 3 78 mg and 79 μg respectively The VMA excretion per kg of body weight shows a tendency to decrease with increasing age (to 2—4 y and to 4—6 y $p<0\,01$ to 6—8 y and 12—16 y $p<0\,01$)

The night time (12 hrs) excretion of VMA was in different age groups 71—75 % of that of daytime ($p<0\,05$)

A remarkable increase in the VMA excretion (ad 33 and 35 4 mg/24 hrs) was shown in two patients with neuroblastoma The changes in clinical condition are reflected in the VMA excretion The VMA excretion was increased in severe infections and in patients with cardiac insufficiency The highest excretion 531 μg/kg appeared in a seven month-old patient with severe cardiac insufficiency and pneumonia Still only few patients in these conditions showed a VMA excretion of over 200 μg/kg The lowest VMA excretion under 10 μg/kg was seen in a 15-year-old boy with chorea minor

The vanilmandelic acid excretion was determined according to a micromodification (Pekkarinen and Hakulinen) of the method of Pisano *et al* (1962) in 0.8 ml urine

Pisano J J J R Crout and D Abraham, *Clin chim Acta* 1962 7 285

Hamberg U (Department of Biochemistry University of Helsinki Finland) VASOACTIVE PEPTIDE RELEASE IN SPONTANEOUSLY LYTIC PLASMA

Previous studies indicated that bradykinin can be released in human plasma by a spontaneously activated protease after treatment to destroy kininase and protease inhibitors Simultaneous generation of plasmin activity occurs with human bovine and rat plasma (Hamberg 1959) Similar results were obtained when activation was performed with urokinase and streptokinase Recent attention has been drawn to this possible relationship between the mechanisms of vasoactive peptide release and fibrinolytic activation Holemans (1965 1966) obtained the release of plasminogen activator with vasoactive peptides from the vessel walls of dog kidney by blood free perfusion and an enhanced fibrinolytic activity of plasma euglobulins in dogs after intravenous injection

The present work deals with the active peptide content in human plasma obtained post mortem from individual cases of sudden death The influence of spontaneously developed lysis following the blood clotting upon the stores of normally bound vasoactive peptides was investigated Plasma was incoagulable with 5—10 N I H units of thrombin and contained a plasminogen activator a free protease with action on fibrin (Hamberg 1966) or neither of these fibrinolytic components in a free state

The release of bradykinin like peptide was effected with *Bothrops* enzyme trypsin or by utilizing the endogenous plasmin Fibrinopeptides potentiating the effect of bradykinin on smooth muscle (Osbahr *et al* 1964) were removed by dialysis before standardization With various types of substrate preparations release of smooth muscle contracting peptide was attained in all cases tested however to a lesser degree compared to normal plasma in samples containing free plasminogen activator and protease Although kininase activity was comparable to normal an endogenous depletion of the peptide stores could not be shown in these strongly lytic plasma Isolation of the plasminogen activator showed similarities with the fibrinolytic tissue activator and differences by molecular weight from the activator formed in human plasma with streptokinase

Hamberg U Fibrinolytic activation and bradykinin release *Biochim Acta* 1959.36.296—298

Hamberg U Separation of plasminogen activators from normal and lytic human plasma *Life Sciences* 1966.5 731—741

Holemans R Origin and stability of plasminogen activator Abstr *Xth congr europ soc haematol* 1965 49

Holemans R The fibrinolysin system *Feder Proc* 1966 25 100—101

Osbahr A J J A Gladner and K Laki Studies on the physiological activity of the peptide released during the fibrinogen fibrin conversion *Biochim biophys Acta (Amst)* 1964.86.535—542

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Hamberger B (Department of Histology Karolinska Institutet Stockholm Sweden) UPTAKE OF CATECHOLAMINES IN RAT BRAIN SLICES AND ITS INHIBITION BY DRUGS

Incubation of brain slices from reserpine pretreated rats in a physiological medium containing catecholamines results in an uptake in the normally catecholamine-containing nerve terminals (Hamberger and Masuoka 1965) which was revealed in the fluorescence microscope This uptake mechanism is energy-dependant and concentrates the catecholamines very efficiently Several drugs were tested on their ability to inhibit this uptake especially in the central nervous system The drugs were either given in vivo or added to the medium before the catecholamines Desipramine and protryptiline effectively inhibit uptake in the noradrenaline nerve terminals and non terminal axons while (+) —amfetamine blocks the uptake in both the noradrenaline and dopamine nerve terminals and non terminal axons The data suggest that the uptake in the noradrenaline and dopamine neuron systems are different

Hamberger B and D Masuoka Localization of catecholamine uptake in rat brain slices *Acta pharmacol (Kbh)* 1965.22 363—368

XII Scandinavian Congress of Physiology Turku 1966
Acta physiol scand 1966.68 Suppl 277

Hauge A., P K M Lunde and H A Waaler (Institute of Physiology University of Oslo Norway) EFFECTS OF SOME VASOACTIVE SUBSTANCES ON VASCULAR RESISTANCE AND VASCULAR CAPACITY IN ISOLATED BLOOD-PERFUSED LUNGS

In the evaluation of the effects of vasoactive substances in the pulmonary circulation the main emphasis has usually been on changes in vascular resistance whereas possible effects on the vascular capacity have been studied to a lesser extent (Aviado 1960) We have attempted to evaluate the effects of some vasoactive substances on pulmonary vascular capacity and on the vascular resistance in isolated rabbit lungs perfused at constant volume inflow (Hauge Lunde and Waaler 1966)

On pulmonary arterial injections adrenaline (1—50 μg) usually caused a decrease and noradrenaline (10—50 μg) some increase in the pulmonary vascular resistance (PVR) As judged by changes in the weight of the preparation which was followed continuously both drugs provoked a reduction in total vascular capacity by some 4—8 % This marked effect and also the PVR increasing effect of noradrenaline were eliminated by adding the α inhibitor phentolamine (Regitin®) 1μg/ml of blood to the perfusate Addition of the β-inhibitor propranolol (Inderal®) 0.2 μg/ml of blood eliminated the PVR reducing effect of adrenaline but did not alter the capacity reducing effect of the catecholamines

Prostaglandin (PGE_1) usually caused a PVR reduction of the same size as that caused by adrenaline but only small capacity changes however Bradykinin injections (1—10 μg) which caused marked PVR increases gave very small capacity effects only

Thus for the drugs tested only the catecholamines caused marked changes in vascular capacity on arterial injection This capacity effect was mediated via α-receptors and there was apparently a dissociation between the α-effect of the catecholamines upon pulmonary resistance and that on the capacitance vessels The resistance and capacitance vessels responding to catecholamines in these lungs must be different ones the former probably located mainly at precapillary sites

Aviado D M *Physiol Rev* 1960.12 159—239
Hauge A P K M Lunde and H A Waaler *Acta physiol scand* 1966.66 226—240

Hauge A P K M Lunde B A Waaler and L Walloe (Institute of Physiology University of Oslo Norway) THE INHIBITORY EFFECT OF VARIOUS PHENOLS UPON ATP INDUCED VASOCONSTRICTION IN ISOLATED PERFUSED LUNGS

In an isolated blood perfused rabbit lung preparation marked vasoconstriction usually developed from 10 min to 2 hr after the start of the perfusion as shown by Hauge Lunde and Waaler (1966) During the same period the reaction of the pulmonary vascular bed to a certain dose of injected ATP changed from dilatation towards marked constriction The spontaneously developing vasoconstriction and the vasoconstriction caused by injected ATP were both inhibited by the addition to the perfusate of tricresol This inhibition of ATP induced vasoconstriction has been further analysed Of the three cresol (mono-methyl phenol) isomers o-cresol and p-cresol were more efficient inhibitors of ATP induced constriction than was m-cresol The tri-cresol preparation originally used has been found however to contain a considerable amount of xylenols (di methyl phenols) in addition to cresol isomers and phenol Of the xylenols 2 4-xylenol has been tested and found to have an effect considerably more marked than that of cresols in inhibiting ATP induced vasoconstriction in this lung preparation

Hauge A P K M Lunde and B A Waaler *Acta physiol scand* 1966.66 226—240

Hedqvist P and L. Stjarne (Department of Physiology Karolinska Institutet Stockholm Sweden) THE ROLE OF THE RECAPTURE MECHANISM IN NORADRENERGIC NERVES

Noradrenergic nerves are known to synthezise noradrenaline (NA) from tyrosine In the heart the NA synthesis rate has been estimated to 0 1—0,2 μg/g/hour As a result of nerve stimulation NA is lost by increased overflow into the circulation Since the organ content of NA remains unchanged except at excessive stimulation this increased loss must be balanced by a rise of NA synthesis Recent results indicate that the NA synthesis rate in certain tissues can be increased 3—4-fold as a result of nerve stimulation To what extent NA released from the axon is recaptured and reused is not yet known although many authors have suggested that the recapture mechanism plays an important role in maintaining the transmitter stores

Nerve stimulation of the isolated perfused cat or dog spleen was found to cause a moderate increase of NA outflow However administration of phenoxybenzamine (PBA) to the perfusion fluid caused a large potentiation of the NA overflow response to nerve stimulation PBA has been shown to prevent uptake of circulating NA into the sympathetic nerve terminals and should thus probably also prevent re uptake of previously released NA Analysis of the NA content of the spleen showed that after PBA administration nerve stimulation resulted in depletion of the major part of the NA in the organ while nerve stimulation without PBA caused no decline or only a moderate decline in the transmitter content

The present results indicate that the organ transmitter stores in the absence of efficient NA recapture would be depleted within 10 to 15 minutes even if NA synthesis is accelerated 3—4 fold Thus recapture of the bulk of the NA released by nerve stimulation appears to be the dominating factor in maintaining the transmitter stores

Heikkinen E, M Valavaara and E Kulonen (Department of Medical Chemistry University of Turku Finland) THE MATURATION OF COLLAGEN *IN VITRO*

L-proline ^3H was injected intraperitoneally into newborn rats After four hours the rats were killed and the skins removed immediately cleaned cut into slices and pooled Samples from the pool were incubated in glucose-containing Krebs Ringer phosphate pH 7.4 for 2 4 6 and 8 hrs in an atmosphere of 95 % O_2 — 5 % CO_2 The incubation was stopped by chilling the samples which were homogenized into 0.45 M NaCl solution Neutral salt soluble acid soluble and insoluble collagen fractions were prepared by consecutive extractions (Heikkinen and Kulonen) The specific activity of hydroxyproline was determined according to Juva and Prockop

During the incubation of 8 hrs the specific activity of neutral salt soluble collagen decreased from 3240 cpm to 2290 cpm/μmole hydroxyproline but simultaenously the activity of acid soluble collagen rose from 160 cpm to 350 cpm/μmole hydroxyproline and the activity of insoluble collagen from 160 cpm to 230 cpm/μmole hydroxyproline The absolute amount of neutral salt soluble collagen decreased and the amount of the other fractions increased during the incubation

The rate of maturation was not affected by anaerobic conditions but freezing and thawing of the slices retarded it With the skin slices from young rats the maturation was found to be faster than with the preparations from old rats

These results suggest that cellular activity is necessary for the maturation of soluble collagens into insoluble form (Supported by U S Department of Agriculture Foreign Research and Technical Programs Division)

Heikkinen E and E Kulonen *Acta physiol scand* In press
Juva K and D J Prockop *Analyt Biochem* In press

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Acta physiol scand 1966 68 Suppl 277

Henning M (Department of Pharmacology University of Goteborg Sweden) URINARY EXCRETION OF CATECHOLAMINES AFTER RESERPINE TREATMENT

The urinary excretion in rats of noradrenaline (NA) after surgical removal of the adrenal medulla is 0.26 μg per kg body weight and hour (s.e.m = 0.019 n = 39) and that of adrenaline (A) is 0.03 μg/kg hour (s.e.m = 0.007 n = 39) Reserpine injection (10 mg/kg intraperitoneally) lowers the amount of NA excreted to below 20 % of the normal after 24 hours At this time the amount of A excreted shows a considerable increase After 48—72 hours the excretion of NA rapidly increases to about 50 % of the normal Re-injection of reserpine (1 mg/kg intraperitoneally) 72 hours after the first dose again lowers the excretion of NA to below 20 % with a less pronounced peak in the amount of A 48—72 hours after the re-injection there is again a rapid increase in the NA excretion to about 50 % The NA in sympathetically innervated tissues (heart spleen skeletal muscle) is virtually completely lost 24 hours after the first injection of reserpine and shows no significant restoration when the excretion of NA in the urine increases towards control values

After reserpine injection into cats the nerve function returns concomitantly with a partial recovery of the ^{3}H NA uptake in the nictitating membrane while the tissues are still depleted of NA The findings agree with the view that an intact adrenergic transmission is dependent on the ability of the storage mechanism to incorporate NA in a small fraction immediately important for the function

Henschen A and U Soderberg (Blood Coagulation Research Department and Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden) MEASUREMENT OF NET EXCHANGE OF FREE AMINO ACIDS BETWEEN BLOOD AND CEREBRAL CORTEX

In a study of the relation of amino acid metabolism to electrical activity of the brain the electroencephalogram (EEG) blood flow and arterio-venous differences of free amino acids have been measured in the cerebral cortex of cats Two types of preparation have been investigated Both employ a large cortical area either isolated from the rest of the brain or with intact nervous connexions The operation was performed in two stages with an interval of 24 hours In the first in Nembutal anaesthesia the isolated cortex preparation was made all draining veins except the superior sagittal sinus were interrupted and EEG electrodes mounted epidurally In the second stage the sagittal sinus was cannulated for recording the rate of venous outflow and for collection of venous blood The technique was designed to avoid contamination with extra-cerebral blood Blood loss through anastomoses did not occur Arterial and sagittal sinus blood were collected at equal rate and frozen immediately Blood drawn for analysis was replaced by whole blood blood cell suspension or dextran The free amino acids in whole blood or plasma were separated after picric acid precipitation and measured quantitatively according to Moore Spackman and Stein

Although the blood levels of several amino acids are high arteriovenous concentration differences were indeed measurable an indication of a considerable net exchange between the blood and the brain The differences varied with the cortical activity but not always with the same time course Thus the effect of a tetanic stimulation could be traced in the pattern of exchange of amino acids long after the recovery of the EEG activity Worthy of note is a considerable output of glycine and alanine from the brain at a rate that has to be taken into account also in calculations of the energy metabolism of the brain

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Hill R. J (Department of Biochemistry University of Aarhus Denmark)
SOME THEORETICAL AND PRACTICAL STUDIES ON QUANTITATIVE IMMUNOELECTROPHORESIS

Oudin (1952) and later other workers (Oakley and Fulthorpe 1953 Augustin *et al* 1958) have developed methods for the quantitative determination of antigens by immunodiffusion in gels Augustin *et al* (1955) and Spiers and Augustin (1958) have given a rigorous mathematical analysis of the one-dimensional Oudin method while others have developed mathematical theories for linear (Engelberg 1959) and radial double diffusion (Korngold and v Leeuwen 1957 Ouchterlony 1958) The mathematical treatments of double diffusion have not taken into account the effect on diffusion of antigen antibody reaction Afonso (1964 1966) developed a two-dimensional quantitative immunoelectrophoretic method for the analysis of serum proteins but gave no mathematical treatment of the method

The present work develops the Afonso technique theoretically and practically treating the problem as one of two-dimensional diffusion from a disc source of antigen into a sheet of agarose impregnated with antibody Reaction of the antigen with antibody is corrected for assuming the kinetics of the reaction to follow (i) Langmuir and (ii) Freundlich adsorption isotherms

Experimental results are given showing that by gradually reducing the antibody concentration in the agarose in different experiments it is possible to determine the diffusion constant of the antigen at infinite antibody dilution This value of the diffusion constant is related to the true diffusion constant Experimental results are also given showing how diffusion coefficients are measured using HSA labelled with ^{131}I as the antigen Programming of a Gier computor for interpretation of these results is also described as is the apparatus used for immunodiffusion and electrophoresis

Afonso E *Clin chim Acta* 1964 *10* 114
Afonso E *Clin chim Acta* 1966 *13* 107
Augustin R, B J Hayward and J A Spiers *Immunology* 1955 *1* 67
Engelberg N *J Immunol* 1959 *82* 467
Korngold L and G van Leeuwen *J Immunol* 1957 *78* 172
Oakley C L and A J Fulthorpe *J Path Bact* 1953 *65* 49
Ouchterlony O *Progr Allergy* 1958 *5* 1
Oudin J *Meth med Res* 1952 *5* 335

Hint H K. Pavek and O Tangen (Research Division Pharmacia AB Uppsala Sweden) STUDIES ON CAPILLARY PORE SIZE BY ESTIMATION OF THE MOLECULAR WEIGHT DISTRIBUTION OF DEXTRAN IN EXTRAVASCULAR TISSUES

Rabbits were given intravenous injections of dextran with rather wide molecular weight distribution (about 10 000 to 2 000 000) Some hours later the animals were anesthetized the central arteries of the ears cannulated and the ears perfused with saline to rinse the vessels from blood and dextran The animals were then sacrificed the ears cut off and dextran isolated from the tissues

The molecular weight distribution of dextran molecules can be estimated by gelfiltration methods in solutions containing less than 1 mg of dextran substance

Comparison of the molecular weight distribution of the infused dextran with the molecular weight distribution of dextran isolated from tissues revealed no evidence for the presence of a certain limiting pore size in connective tissue capillary membranes

Hirvonen L. T Peltonen M Arstila and P Makela (Cardiorespiratory Research Unit Department of Physiology University of Turku Finland)
RESPIRATION CIRCULATION AND THE ALIMENTARY CANAL AT BIRTH

A black—white X ray film The recordings were performed with an Arriflex 35 mm movie camera connected with a Philips image intensifier on lambs with a rate of 16 to 48 exposures per second The following phenomena are demonstrated the first breath neonatal breathing with bronchography flow of radiopaque material through the foetal shunts before and after the first breath angiocardiography at different ages sucking swallowing and motility of the gastrointestinal tract of the new born animal

Peltonen T and L Hirvonen Experimental Studies on Fetal and Neonatal Circulation *Acta paediat (Uppsala)* 1965 Suppl 161

Hopsu V O Hanninen K Juva E Kaarsalo M Lehtinen J Maatela C G Nordstrom V Nanto and B Laurent (Departments of Anatomy Medical Chemistry Medical Microbiology Physiology and Clinical Chemistry University of Turku Finland) STUDIES ON HAPTOGLOBIN SYNTHESIS IN THE RAT

A two- to threefold increase in rat serum haptoglobin level was induced within 24 hours by a single subcutaneous (sc) injection of hexane cyclohexane methylcyclohexane α pinene β pienene or turpentine The turpentine contained 68 % (v/v) of α pinene The dose of hydrocarbons administered varied from 0.5 to 4.0 ml/kg of body weight Increased haptoglobin values were observed after sc injection of hexane in adrenalectomized rats Simultaneous sc injection of carbon tetrachloride inhibited the effect of hexane Intramuscular on intraperitoneal administration of hexane caused similar increases in serum haptoglobin values as when injected subcutaneously Intramuscular or intraperitoneal administration of rat skin extracts injected in doses containing 50—150 mg of dry matter doubled the serum haptoglobin values in 24 hours

By experiments on rats we have thus been able to confirm and add a few data to the results obtained by previous investigators from work done mainly on rabbits (Review by 1) It seems probable that the liver is the sole source of haptoglobin production in mammals The peak values of rabbit serum haptoglobin occur in less than 24 hours after intravenous injection of a tissue extract while the maximum effect caused by sc turpentine injection appears after about 48 hours (Mouray et al 1964) This might be used as evidence in favour of a hypothesis according to which the increase in haptoglobin synthesis may result from a liver stimulating factor released from damaged tissue (Mouray 1966 Mouray et al 1964) A direct effect of hydrocarbons on the liver however should also be considered Experiments *in vitro* are now under work to test the latter possibility Histochemical techniques are being applied to correlate the increase in serum haptoglobin with enzyme activities of the liver

Mouray H Biosynthese de l'haptoglobine chez le lapin *Thesis* Paris 1966
Mouray H J Moretti and M F Jayle *C R Acad Sci (Paris)* 1964 259 2721

Huovinen J A and B E Gustafsson (Department of Medical Chemistry University of Helsinki Finland and Department for Germfree Research Karolinska Institutet Stockholm Sweden) UTILIZATION OF INORGANIC SULPHATE SULPHITE AND SULPHIDE FOR SULPHUR AMINO ACID BIOSYNTHESIS IN GERMFREE AND CONVENTIONAL RATS

Incorporation of ^{35}S from labelled sodium sulphate sulphite and sulphide into cysteine and methionine in germfree and conventional rats was investigated Six to seven weeks old germfree rats reared according to Gustafsson (1948 1959) and conventional animals of the same strain and age were used Each sulphur compound was administered into five germfree and five conventional rats 300 μC of sulphate and sulphite and 60 μC of sulphide were injected intraperitoneally The specific activity of all compounds was 8 mC/mmole After 3 hours the animals were killed

No incorporation of S into isolated cysteine or methionine of serum liver kidney and pancreas was found in germfree animals injected with sulphate or sulphite Low radioactivities of cysteine and methionine were found in conventional animals in sulphate and sulphite groups Ten times higher specific activities of cysteine were found in germfree and conventional rats injected with sulphide Labelling of methionine was detected in conventional rats only

Results obtained in some preliminary experiments *in vitro* were consistent with these findings No incorporation of radioactivity into sulphur amino acids was detectable in homogenates of germfree rat liver kidney and gut when incubated with sulphate or sulphite Labelling of cysteine was observed in all homogenates after incubation with ^{35}S sulphide

These findings show that the germfree rat is able to fix inorganic sulphur at the sulphide level of oxidation into amino acid linkage as cysteine Inorganic sulphate and sulphite are not in detectable amounts used for the synthesis of sulphur amino acids in germfree animals

Gustafsson B E Germfree rearing of rats general technique *Acta pathol microbiol scand* 1948 Suppl 73 1—130
Gustafsson B E Lightweight stainless steel systems for rearing germfree animals *Ann N Y Acad Sci* 1959 78 17—28

Hytönen Y and U-K. Tiihonen (Institute of Physiology University of Helsinki Finland) SUMMATION OF SUBLIMINAL PITCH AND LOUDNESS CHANGES IN AUDITORY DISCRIMINATION OF THE DOG

The phenomenon of intramodal interaction of subliminal changes in different dimensions of a sensory stimulus is a fundamental one in the process of signal detection It has previously been examined on the basis of human experiments in different sense domains (Bergstrom 1964) Animal studies on the subject are encountered by considerable methodological difficulties In the auditory domain only a preliminary study on the rat has been performed (Hytonen 1964) Animal studies add however an interesting phylogenetical aspect to the study of the nature of such integrative functions — Dogs were trained by the method of avoidance conditioning to respond to changes in pitch and loudness of pure tones Differential threshold values of combined pitch and loudness changes versus differential thresholds for each dimension separately indicate quantitatively the interaction between subliminal pitch and loudness changes in the discriminatory process Experiments were carried out on the frequency levels of 700 1 400 2 800 cps and on an intensity level approximately 50 db above 10^{-16} Watt/cm² As in previous human and animal experiments the results show that summation of pitch and loudness in auditory discrimination occurs also in the dog The summation appears to be a function of non linear character

Bergstrom R. M Über das Wahrnehmen der Zeit als Wahrnehmen der Bewegung *Ann Acad Sci fenn A5* 1964 106/2
Hytonen Y On the summation of pitch and loudness in auditory discrimination of the white rat *Ann Acad Sci. fenn A5* 1964 106/6

Hyvarinen J (Institute of Physiology University of Helsinki Finland)
INTENSITY FUNCTIONS OF SPONTANEOUS SERIES OF NEURAL EVENTS

Intensity functions (IF) (Cox 1965) and the corresponding renewal densities (RD) (Cox 1962) have been estimated for extracellularly recorded spontaneous neural impulse sequences from the diencephalic region of rabbits ranging in age from newborn to adult 43 per cent of the 299 studied neurons gave intensity functions of periodic character This periodicity was related mainly to the RD in 41 per cent of the periodic ones In the remaining 59 per cent there was clearly periodic time dependency These results show a smaller percentage of periodicity than was found in the ED-curves of the selected material of Poggio and Viernstein (1964) No significant differences in the periodic time dependency were found between different nuclei There were however two age groups where the periodic time dependency sharply increased the first at the age of two weeks and the second at the beginning of puberty at the age of ten weeks In 21 neurons that gave both periodic RD and IF the periods differed from each other leading to a phase shift between the curves Cells that showed tendency to rapid bursts of spikes might give IFs with constantly high probability of firing until a sudden drop occurred after the burst duration In other samples the IFs would oscillate around the stabilized RD seemingly at random but often when tested these oscillations show time dependency which is of a non periodic nature

Cox D R. *Renewal theory* London Methuen. 1962 pp 53—55
Cox D R On the estimation of the intensity function of a stationary point process *J R. statist Soc B* 1965 27 332—337
Poggio G F and L J Viernstein Time series analysis of impulse sequences of thalamic somatic sensory neurons *J Neurophysiol* 1964 27 517—545

Häggendal J (Department of Pharmacology University of Göteborg Sweden) CATECHOLAMINES IN HUMAN CEREBROSPINAL FLUID

The levels of noradrenaline (NA) in the cerebrospinal fluid (CSF) have been estimated fluorimetrically according to the principles of the tri hydroxyindole reaction in patients mainly from a mental hospital (Dencker Haggendal and Ilves—Haggendal 1966) In 68 of 72 cases investigated the levels of free NA were 0.5±0.03 $\mu g/l$ (mean ± s.e.m) Typical spectra of NA were obtained in several cases however not in all The levels were so low in some cases that the estimation appears to have been performed at the limit of the sensitivity of the estimation procedure

In the remaining four cases much higher values were found 2.3 2.8 16.8 and 2.3 $\mu g/l$ — in the last case mainly adrenaline

Of the CSF samples 22 were subjected to acid hydrolysis The NA levels were increased in 19 cases and as a rule more evident NA spectra were obtained In the cases where the levels of free NA were 2.8 $\mu g/l$ after acid hydrolysis a value of 2.9 $\mu g/l$ was obtained. In the other 21 cases the level of free NA was 0.4±0.04 and after acid hydrolysis 0.6±0.07 $\mu g/l$ (mean ± s.e.m) Thus conjugated NA seemed to be present in CSF as in human urine and blood plasma

In human brain tissue no increased NA values were found after acid hydrolysis

After oral administration of NA the levels of conjugated NA in blood plasma and in CSF were higher than normally found

The efficiency of the blood CSF and blood brain barriers to cate cholamines will be discussed with respect to these results and some histochemical and biochemical evidence obtained from rabbit brain after dopamine and NA administration i v or i p (Dahlstrom Haggendal and Waldeck 1966)

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Hakkinen H M and E Kulonen (Research Laboratories of the State Alcohol Monopoly (Alko) Helsinki Finland) EFFECT OF ETHANOL ON THE UTILIZATION OF GLUCOSE *IN VITRO* BY THE SOLUBLE PROTEIN FRACTION OF RAT BRAIN

In the standard experiment fasting rats received 5.5 g/kg ethanol by a stomach tube and the control rats the same volume of water After one hour the rats were killed the brains dissected and homogenized with Potter—Elvehjem s apparatus into KCl solution buffered with phosphate to pH 7 4 After centrifugation at 100 000 g for 60 min the soluble protein fraction was obtained with gel filtration through a Sephadex G-25 column This fraction was diluted with KCl—Na phosphate solution pH 7 4 and incubated for 30—60 min at +37 C with glucose as the substrate in the presence of NAD^+ ATP Mg^{++} fumarate and cytochrome c as suggested by DiPietro and Weinhouse (1959) After the incubation period glucose lactic acid and in some cases pyruvic acid were determined from the incubation mixture applying enzymatic methods Several experimental variations were tested Instead of glucose also glucose 1 phosphate glucose 6-phosphate glycogen fructose mannose and galactose were used as substrates

This system converted about 40—50 % of the utilized glucose to lactic acid and the proportion was not affected by the administration of ethanol When the soluble protein fraction had been prepared from ethanol intoxicated rats 1—3 h after the ingestion of ethanol the consumption of glucose and consequently the formation of lactic acid increased by 20—25 % Similar effect was observed also when glycogen glucose 1 phosphate or fructose were used as substrates but not in the conversion of glucose-6-phosphate to lactic acid Ethanol intoxication seems thus to affect the phosphorylation of hexoses and the phosphoglucomutase reaction in the brain

DiPietro D and S Weinhouse Glucose oxidation in rat brain slices and homogenates *Arch Biochem Biophys* 1959.80.268—282

Hakkinen I P T (Department of Physiology University of Turku Finland) GASTRIC SULPHOMUCOPROTEINS IN NORMAL PRECANCEROUS AND CANCEROUS GASTRIC JUICE

Empirically and partly on morphological grounds the changes in the mucous lining of the stomach have earlier been called and classified as precancerous In the present work the product of the epitelial cells of the stomach the group of sulphomucoproteins has been studied By characterising these in connection with the various changes in the mucous membrane molecular classification has been attempted The chemical methods proved least effective and sensitive The information given by them is however the best evidence of qualitative differences although they could not be used in the diagnostic sense Various physico-chemical methods such as analytical ultracentrifugation and zone electrophoresis gave instead a clear picture of polydispersity which appeared in various degrees in precancerous states as in pernicious anemia and which reached its peak in some cases of carcinoma The best and most sensitive way of classifying this atypicalness of sulphomucoproteins proved to de the immunochemical method Based on this it has indeed been possible on one hand to differentiate precancerous states from the normal and carcinoma from these This immunochemical method has been described elsewhere (Hakkinen 1966)

Hakkinen I P T An immunochemical method for detecting carcinomatous secretion from human gastric juice *Scand J Gastroenterology* 1966 In press

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Häkkinen V (Institute of Physiology University of Helsinki Finland)
EFFECT OF HYPNOTICALLY INDUCED MUSCLE TONUS ON AUDITORY DISCRIMINATION

The afferent inflow brought about by muscular activity and tonus has been shown to induce cerebral arousal and increased vigilance (Motokizawa *et al* 1964 Bloch *et al* 1965) It is understandable that through this mechanism afferent muscular inflow also influences sensory perception This has been dealt with earlier by Simpson 1937 Krus *et al* 1953 1958 and Starr 1964 Both facilitatory and inhibitory influences have been reported The present investigation was designed to study the effect of muscle tonus on auditory discrimination in man The muscle tonus was obtained by hypnotic induction During moderate localized muscle tonus an increase in discrimination was obtained though hypnosis as such does not seem to alter discrimination

Bloch M M Valat et J C Roy *J Physiol (Paris)* 1965 *57* 561—562
Krus D M H Werner and S Wapner *Amer J Psychol* 1953 *66* 603—608
Krus [redacted] M S Wapner and H Werner *Amer J Psychol* 1958 *71* 395—398
Motokizawa F and B Fujimori *Jap J Physiol* 1964 *14* 341—353
Simpson R M *Ph D thesis* Northwestern University 1937
Starr A *Exp Neurol* 1964 *10* 191—204

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Hanninen O (Department of Physiology University of Turku Finland)

COUPLED INDUCTIONS IN THE GLUCURONIC ACID PATHWAY BY THE ADMINISTRATION OF CINCHOPHEN IN THE RAT

The steps in metabolic pathways are sometimes grouped at the genetic level in order to ensure their efficient function as exemplified by the histidine enzymes of *E coli* In the present study the activities of the different enzymes of the glucuronic acid pathway were studied to find out enzyme groups induced simultaneously after intragastric administrations of one mmole of cinchophen in adult male rats

The activity of uridine diphosphate glucose dehydrogenase and uridine diphosphate glucuronic acid transferase increased 1.5—2 fold in the kidney and liver after two administrations when analysed 24 hours after the last dose The increase reached its maximum in both cases within six administrations No increase or only a slight increase was observed in the gastrointestinal mucous membrane But if the analyses were carried out 48 hours after the last administration of the drug the induction was also in the gastrointestinal mucous membrane equal to that in the kidney and liver This may reveal a release of inhibition due to the high concentrations of cinchophen or its metabolites The activity of glucuronolactone dehydrogenase increased parallelly to the two earlier described enzymes in the liver and kidney But the activity of β glucuronidase uronolactonase aldonolactonase glucuronolactone (and glucuronic acid) reductase remained practically unchanged during the cinchophen treatment

The early steps of the pathway could thus be induced by the administration of the drug and also the glucuronolactone dehydrogenase an enzyme rather remote to the first two Glucuronolactone dehydrogenase is necessary for the synthesis of glucarolactones Glucaro-1 4 lactone is generally known to be a powerful and specific inhibitor of β glucuronidase which splits the β D glucopyranosiduronic acids The induction of glucuronolactone dehydrogenase increases thus the efficiency of the defence mechanisms providing an increased synthesis of a feedback inhibitor of the free glucuronic acid release It is possible on the basis of the induction kinetics that these three enzymes form a genetically linked group

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Hanninen O K Alanen and K Hartiala (Department of Physiology University of Turku Finland) **THE LEVELS OF URIDINE DIPHOSPHATE GLUCOSE DEHYDROGENASE AND UDPG IN THE GASTROINTESTINAL MUCOUS MEMBRANE**

The activity of uridine diphosphate glucose dehydrogenase was studied in the mucous membrane of the different gastrointestinal segments in the rat and in the different layers of the duodenal 10 cm segment in the dog The UDPG content in the small intestine mucosa was determined after trichloroacetic acid perfusions in the rat The methods have been described earlier (Hanninen 1966)

The enzyme activity decreased expotentially in the mucous membrane along the first half of the small intestine in rats but remained almost unchanged in the last half It again increased in caecum to the level of the first 5 cm segment of the small intestine remaining high also in the proximal large intestine The glandular stomach and the distal large intestine were similar to the ileal segments For comparison the enzyme activity in the liver was about half of the maximal activity of the small intestine The intestinal contents were inactive The enzyme activity can be induced by peroral administration of cinchophen also in other parts of the gastrointestinal tract like in the glandular stomach and duodenum The enzyme activity in the 200 μ successive frozen sections of the dog duodenal wall was low in specimens containing muscle fibers After reaching a 2 mm depth the activity practically disappeared The activity of the entire wall preparation was about one fourth of the activity in the most superficial slices

The UDPG content was found to be about two-thirds of the content in liver and was the same in the five analysed segments of the rat small intestine

The activity of the UDPG dehydrogenase behaves quite differently from the activities of several enzymes transferring phosphate residues reported earlier by us The enzyme provides glucuronic acid for the β D-glucopyranosiduronic acid and polysaccharide biosynthesis According to our observations the mucosal formation of conjugates decreases along the small intestine being low also in the large intestine The synthesis of mucus is however high also in the lower parts of the intestine

Hanninen O Effect of salicylamide administration on D glucuronic acid metabolism in the rat *Ann Acad Sci fenn* A5 *1966* 123

Hanninen, O and K. Hartiala (Department of Physiology University of Turku Finland) THE ACTION OF CINCHOPHEN ON THE ENZYMES OF GLUCURONIC ACID PATHWAY *IN VITRO*

It has been suggested that the induction in the glucuronic acid pathway at least in some steps may be due to a depression caused by the inhibitory action of the inducing compound (Hanninen 1966) Peroral administration of cinchophen causes a high induction of some steps in the pathway

Cinchophen (10^{-4} M) has been found to inhibit *in vitro* rat liver uridine diphosphate glucose dehydrogenase hexonate dehydrogenase and glucuronolactone dehydrogenase preparations The mechanism of the inhibition seems to be mainly the competition with the coenzyme β-Glucuronidase uronolactonase and aldonolactonase are not affected by the low concentrations of the drug Some of the derivatives of cinchophen seem to be almost equally effective in action on the dehydrogenases (6- 7 and 8 hydroxycinchophens) but 3-hydroxycinchophen exceeds the parent compound 3-hydroxycinchophen is not however formed during the metabolism of cinchophen after intragastric administrations in the rat The susceptibility of dehydrogenases to the different cinhcophen derivatives are in line with the earlier observations on their effect on the rat liver transaminases (Hanninen and Hartiala 1965)

Hanninen O Effect of salicylamide administration on D glucuronic acid metabolism in the rat *Ann Acad Sci fenn* A 5 1966.123

Hanninen O and K Hartiala Inhibition of transaminases by cinchophen and its derivatives *Biochem Pharmacol* 1965.14 1073

Jansson G M Kampp O Lundgren and J Martinson (Department of Physiology University of Goteborg Goteborg Sweden) STUDIES ON THE CIRCULATION OF THE STOMACH

By recording volume and total venous effluent simultaneously the resistance the capacitance and the precapillary sphincter segments of the circulation of the cat stomach were studied Distribution of blood flow within the stomach was ascertained using a modified krypton^{85}-clearance technique

The control capillary filtration coefficient (CFC) was similar to that for intestine Vasodilator drugs increased CFC indicating dilatation of precapillary sphincters and increased capillary surface area Capacitance vessels relaxed increasing the regional blood volume as much as 40 %

Vagal stimulation decreased resistance but had less effect on capacitance CFC however increased more than during a comparable flow increase caused by vasodilator drugs The increased CFC persisted several minutes after cessation of vagal stimulation and may be due to increased capillary permeability

Splanchnic nerve stimulation and infusion of noradrenaline caused vasoconstriction followed by an autoregulatory escape as in the gut (Folkow *et al* 1964) Concomitantly CFC was decreased and remained so during the escape Mean capillary pressure was not significantly changed Regional blood volume was reduced as much as 30 %

The wash-out of krypton^{85} intra arterially injected was monitored by an external scintillation detector and the curve could be resolved into three components (I—III) We propose that the fastest component (I) represents mucosal flow II is muscularis flow and the slowest (III) represents tissues outside the stomach wall Assuming a tissue/blood partition coefficient equal to one components I and II correspond to blood flow values of 60—140 and 10—20 ml/min$\times$100 g respectively

Folkow B D H Lewis O Lundgren S Mellander and I Wallentin *Acta physiol scand* 1964.61 445—457

Jansson G B Lisander and J Martinson (Department of Physiology University of Goteborg Goteborg Sweden) HYPOTHALAMIC CONTROL OF ADRENERGIC OUTFLOW TO THE STOMACH

Structural and physiological evidence suggest that adrenergic neurons can block the parasympathetic cholinergic nerve impulse transmission within the myenteric and submucous plexa of the gastrointestinal tract (Norberg 1964 Jansson and Martinson 1966) These neurons may be under influence from central nervous structures In acute experiments on adrenalectomized cats vagal and myogenic responses could be separately evaluated when gastric motility was studied under isotonic volumetric conditions The cats were stereotactically stimulated in the hypothalamic defence area (cf Abrahams Hilton Zbrozyna 1960) and the nearby sympatho inhibitory area (Folkow Johansson Öberg 1959)

Stimulation in the defence area besides eliciting the characteristic cardiovascular changes activated adrenergic neurons which promptly inhibit vagal cholinergic impulse transmission On the other hand stimulation within the inhibitor area inhibited the adrenergic neurons to the stomach thus releasing the vagal impulse transmission which resulted in augmented excitatory responses of the stomach to vagal stimulation

Abrahams V C S M Hilton and A Zbrozyna *J Physiol* (Lond) 1960 *154* 491
Folkow B B Johansson and B Öberg *Acta physiol scand* 1959 *47* 262
Jansson G and J Martinson *Acta physiol scand* In press
Norberg K A *Int J Neuropharmacol* 1964 *3* 379—382

Jansson G B Lisander and J Martinson (Department of Physiology University of Goteborg Goteborg Sweden) RELAXATION OF THE STOMACH ELICITED VIA VAGUS NERVES FROM CENTRAL NERVOUS STRUCTURES

Electrical stimulation of the peripheral end of the cut vagus nerves can activate a set of nerve fibres that profoundly relaxes the corpus fundus of the stomach (Jansson and Martinson 1965) It seemed likely that these fibres could be excited from central nervous structures Electrical stimulation within well defined areas in the central nervous system was performed utilizing the Horsley—Clarke technique on cats Relaxation of the stomach measured with an isotonic volumetric technique was observed when stimulation was performed within the ventral postero-medial nucleus (VPM) in the thalamus within the mamillary body and vagus nucleus The relaxation was mediated *via* the vagus nerves and it had the same properties as the relaxation which could be elicited by vagal stimulation in the neck thus the relaxation was resistant to both atropine and guanethidine and the relaxation was accomplished by changing the myogenic tone of the corpus and fundus and not by inhibiting pre-existing excitatory vagal motor control as do adrenergic fibres to the stomach (Jansson *et al* 1966)

The fact that this response can be elicited from the thalamic relay nucleus for taste suggests the involvement of the vagal relaxatory fibres in the receptive relaxation occurring during food intake

Jansson G and J Martinson *Acta physiol scand* 1965.63.351—357
Jansson G B Lisander and J Martinson *Acta physiol scand* 1966 *68* Suppl 277 92

Johansson G L. Laitinen and P Sipponen (Stereotaxic Laboratory of the Neurosurgical Clinic Helsinki Finland) REGIONAL BLOOD FLOW RECORDED BY AN IMPEDANCE TECHNIQUE

We have used impedance and phase angle recording for locating deep cerebral structures by means of a monopolar roving electrode introduced during stereotaxic operations When the apparatus was set to measure more delicate changes in impedance an impedance magnitude pulsation corresponding to the heart rate could be recorded The time lag between the QRS-complex and the impedance pulse wave was approximately 150 msec The impedance pulse wave consisted of a steep impedance decrease corresponding to the systole and of a slower often dicrotic rise in impedance Compression of the jugular veins caused a slow decrease of the impedance with a simultaneous diminution of the pulse amplitude Compression of the ipsilateral carotid artery in the neck diminished the pulse amplitude with a slight simultaneous rise of the base line Respiration caused a rhythmic fluctuation of the base line Increased intracranial pressure and changes in arterial CO_2 pressure influenced the impedance base line and pulsation

Similar pulsations were recorded with implanted electrodes from subcutaneous tissue of the forearm

The impedance magnitude pulsation is due to local blood flow variations This method may be a useful aid in measuring regional blood flow especially in the brain

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Johnsson G A Norrby L. Solvell and B Ablad (Department of Pharmacology University of Goteborg Sweden) STUDIES ON THE POTENCY AND TIME EFFECT RELATION OF BETA ADRENERGIC ANTAGONISTS IN MAN

The beta adrenergic blocking agents propranolol and H 56/28 (1 (o-allyl phenoxy) isopropylamino-2 propanol hydrochloride) have been studied with regard to intrinsic beta adrenergic stimulating effect beta blocking effect rate of onset and duration in man The effect of the drugs on heart rate arterial blood pressure forearm blood flow and the interference with the effect of repeated infusions of isopropylnoradrenaline on the same parameters have been compared

The results indicate that H 56/28 had a weak positive chronotropic effect on the heart while propranolol was devoid of such an effect

Propranolol and H 56/28 both antagonized the positive chronotropic and inotropic effects of isopropylnoradrenaline as well as its vaso-dilating effect Propranolol was generally a more potent antagonist than H 56/28

When given orally the beta adrenergic blocking effect of the two drugs was significant within 30 minutes The effect reached its maximum between one and three hours and persisted for more than four hours after the administration of the drug

Studies with the two optical isomers of H 56/28 indicated that the beta adrenergic blocking activity resided in the laevo-isomer The potency of this isomer was of the same magnitude as that of propranolol

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Jonsson G and M Ritzén (Institute of Histology Karolinska Institutet Stockholm Sweden) HISTOCHEMICAL DEMONSTRATION OF METARAMINOL

m Hydroxyphenylethylamines such as m hydroxytyramine m hydroxy amphetamine and m hydroxynorephedrine (metaraminol) in a dried protein layer condense with formaldehyde forming fluorescent 6-hydroxy 3 4-dihydroisoquinolines (Corrodi and Jonsson 1966) The reaction is quite analogous to that between formaldehyde and biogenic monoamines used for the histochemical demonstration of these amines according to the method of Falck and Hillarp Using a microspectrophotometric technique it has been possible to demonstrate uptake and accumulation of injected metaraminol and α methyl m tyrosine within the adrenergic neuron with and without reserpine pretreatment Appearance of characteristic blue fluorescence (max at 415 mμ) due to presence of metahydroxy compounds with simultaneous disappearance of the specific catecholamine fluorescence (max at 470 mμ) could be seen to follow administration of metaraminol and α methyl m tyrosine The results support the view that noradrenaline depletion by metahydroxy compounds is produced by an exchange between the amines

The blue fuorescence of the metahydroxy compounds explains earlier failures to visualize these amines intraneuronally with the secondary filters used (strong absorption below 480 mμ)

Corrodi H and G Jonsson *Helv Chim Acta* 1966 49 798

uul Nielsen J (Danmarks Apotekerforenings Kontrollaboratorium Copenhagen Denmark) INVESTIGATIONS ON THE ABSORPTION OF AMINO ACIDS FROM DIFFERENT PROTEIN SOURCES GIVEN PER OS AMINO ACIDS IN BLOOD FROM VENA PORTAE

A pig having a permanent catheter in the vena portae is used for the xperiments

Blood samples are drawn at intervals varying from five minutes up to ne hour in the experimental period after feeding the pig with different roteins The content of free amino acids in the plasma is analysed using utomatic column chromatography

The present results show that the intensity of the absorption from the limentary tract to the portal blood has two maxima after having being ed with natural proteins

The importance of the results is discussed in relation to an effective reatment of protein and amino acid deficiency

Kahri A. (Department of Anatomy University of Helsinki Finland) EFFECTS OF ACTH ON THE FINE STRUCTURE OF CULTURED CORTICAL CELLS OF THE RAT ADRENALS

Effects of ACTH on the fine structure of cultured cortical cells of the foetal (19 to 21-day-old) rats adrenals were studied using a tissue culture method.

With fowl plasma and chicken embryo extract coagulum fragments of the adrenals were cultured on the bottom of plastic dishes (Falcon Plastic Inc 15×60 mm) The medium consisted of 50 per cent Melnick s solution A (Hanks BSS+0.5 per cent lactalbumin hydrolysate) 25 per cent calf serum and 25 per cent amino acid Parker An exceptional feature of the present tissue culture system was that it was open Without replenishment of the medium the cultures were incubated in a humidified air atmosphere at 37 C and the pH adjusted with continuous flow of carbon dioxide gas

0 1 IU/ml of ACTH (Cortrophin®ACTH Organon) was added to the medium of the tissue cultures of the adrenals every day for six days from the 16th cultivation day up to and including the 21st day On the seventh (or 22nd) day from the start of treatment the cultures were fixed and studied with the aid of electron microscopy With phase optics it was seen that the epithelial cells were enlarged and the nucleus somewhat rounded The ultrastructure of the epithelial cells resembling zona glomerulosa cells was not changed The epithelial cells resembling zona intermedia cells were altered into cells resembling zona fasciculata cells The size of the cells increased The chromatin of the nucleus had condensed along the nuclear membrane as coarse clumps The mitochondria were large and their internal structure had become vesicular The amount of smooth-surfaced endoplasmic reticulum had increased and the Golgi saccules were dilated to large vesicles The number of microvilli had increased Thus ACTH had induced transformation of epithelial cells in tissue cultures of adrenals into fasciculata cells which were never found in the untreated controls

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Kallio V., M I Linna and T M Scheinin (Departments of Surgery Internal Medicine and Physiology University of Turku Finland) EXPERIMENTAL STUDIES ON THE INCIDENCE OF VENTRICULAR FIBRILLATION AFTER DIRECT CURRENT DISCHARGE

The first series of 7 mongrel dogs received 540 direct current (DC) discharges in an attempt to assess the relation between energy level of the shock and incidence of ventricular fibrillation (VF) Shocks from 1 to 200 watt seconds were delivered alternately during the downstroke of the R wave safe period and immediately preceding the apex of the T wave vulnerable period

No serious arrhythmias occurred after discharges during the R wave regardless of energy level VF was easily provoked with shocks particularly of low energy (10—50 watt seconds) during the vulnerable period

The second series of 5 dogs received 1205 DC shocks in an evaluation of the influence of digitalis (0 07—0.21 mg/kg body weight) on the occurrence of VF after discharges of 1 to 100 watt-seconds

Digitalization did not appreciably alter the incidence of VF regardless whether shock was given during the safe on the vulnerable period With increasing amounts of digitalis VF became however more frequent after vulnerable period discharges in the very low energy range

Digitalis in amounts exceeding 0 175 mg/kg body weight was poorly tolerated Severe arrhythmias occurred which made correct synchronization of the DC discharge difficult or impossible This may have accounted for the occurrence of a few episodes of VF even after attempted delivery of the countershock during the safe period

Kampp M and O Lundgren (Department of Physiology, University of Goteborg, Sweden) BLOOD FLOW AND FLOW DISTRIBUTION WITHIN THE SMALL INTESTINE OF THE CAT

Blood flow and flow distribution within the small intestine of the cat was studied by monitoring the disappearance of intra-arterially injected krypton^{85}. The experiments were performed on an acutely sympathectomized segment of the jejunum. The γ activity emitted by Kr^{85} was registered externally by a scintillation detector and the β activity by GM tubes placed in the lumen of the gut and/or on the external wall. Venous outflow from the jejunal segment was simultaneously recorded by means of a drop recorder unit.

The multiexponential γ-curve which was observed could be resolved into four components (I—IV) by successively subtracting exponentials as originally proposed by Dobson and Warner (1957). Evidence obtained from the wash-out of the β activity and from experiments with local tracer injections into different layers of the intestine favors the following localizations of the four components. The very slow component (IV, half time value 20—60 min at a venous outflow of 15—50 ml/min$\times$100 g) is located outside the intestinal wall, probably in the perivascular fat of the mesentery. Component III (half time value 4—9 min, about 25 % of the initial activity is distributed to this component) mainly reflects blood flow within the muscularis. Component II (half time value 1—2 min, per cent initial activity 30—40) is located in the mucosa. Assuming a tissue/blood partition coefficient equal to one, components II and III correspond to blood flows of 10—20 and 30—70 ml/min$\times$100 g respectively. The fastest component (I, half time value 0.05—0.20 min, per cent initial activity 35—50) may reflect short circuit of Kr^{85} via countercurrent exchange in villi. Evidence for this hypothesis is presented elsewhere (Kampp and Lundgren 1966).

Dobson E L and G F Warner *Amer J Physiol* 1957 189 269
Kampp M and O Lundgren *Acta physiol scand* 1966.68 Suppl 277 103

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Kampp M and O Lundgren (Department of Physiology University of *Goteborg Sweden*) EVIDENCE FOR COUNTERCURRENT EXCHANGE IN INTESTINAL VILLI

Morphological studies show that non branching arterioles running in the middle of intestinal villi are surrounded by a dense capillary network lying just beneath villi are surrounded by a dense capillary networks is consistent with the possibility of passive countercurrent exchange preferentially of lipid soluble substances since the main direction of capillary flow must be opposite that of the arterioles

To investigate this problem the following experiments were performed on acutely sympathectomized segments of cat jejunum 1 The fastest component of the multiexponential wash-out curve following intra arterially injected krypton^{85} has a half time value of 0 05—0.20 min representing a shunting of the lipid soluble tracer (Kampp and Lundgren 1966) In contrast krypton^{85} wash-out curves of stomach which has no morphological indications of countercurrent exchange in the mucosa do not have any such rapid component

2 *Krypton^{85} deposited between the mucosal surface and a thin diffusion* barrier (Mylar) had a clearance rate not greater than krypton^{85} deposited on the serosal surface despite the greater mucosal blood flow and absorption surface Thus there seems to exist a relative hindrance for the clearance of krypton^{85} from the mucosa

3 Lipid soluble 4-iodoantipyrine I^{131} and water soluble rubidium^{86} were simultaneously infused intra arterially to jejunal segments Within 30 seconds of the start of the infusion the vessels were clamped and the tissue immediately frozen on dry ice The villi were cut transversely into three segments and the tips contained relatively less 4-iodoantipyrine I^{131} than rubidium^{86} when compared to the bases of the villi ($P<0\ 005$ $n=10$) This indicates a predominant cross diffusion of the lipophilic tracer at the bases of the villi

The observations thus support the proposed hypothesis of counter current exchange in small intestine

Kampp M and O Lundgren *Acta physiol scand* 1966 68 Suppl 277 102

Karlmark B (Department of Physiology and Medical Biophysics University of Uppsala Sweden) DOSE RESPONSE RELATIONSHIP FOR ADH AND INHIBITION OF GASTRIC ACID SECRETION

It is well known since the middle of the 19th century that physical exercise is a potent inhibitor of gastric secretion The mechanisms for this inhibition are not yet understood but one of them could be the liberation of the Anti Diuretic Hormone from the posterior lobe of the hypophysis (Verney 1947) Many investigators have shown that extracts of pituitary reduces gastric acid secretion This inhibition takes place with stimulants such as histamin insulin and sham feeding Verney (1947) showed that dogs during emotional stress (e g muscular exercise) reacted with a significant decrease of induced water diuresis This phenomenon was observed after denervation of the kidneys and suprarenal glands The explanation for the antidiuresis was a liberation of a humoral factor After hypophysectomy identical experiments showed that the antidiuretic response was only 5 % Other investigators have shown similar results

I have studied the dose response relation for ADH and inhibition of gastric acid secretion Heidenhain pouch dogs were fed every ten minutes throughout the experiment with 10 g forcemeat and 1.5 g bonedust (Obrink 1954) A constant gastric secretion rate was induced in this way ADH was infused with a constant rate during 40 minutes This time corresponds to the time for physical exercise (treadmill) in other types of experiments

The two most common natural antidiuretic hormones (Lysine ADH and Arginine ADH) were used in synthetic forms The inhibition of gastric secretion was plotted against dosage of ADH ED_{50} thus could be determined Chemical assays of the ADH are not available so the result was compared with the physiological range of the ADH titre obtained from bioassays

With Lysine ADH I have found ED_{50} about 2000 μE/min This may well correspond to the dose which physiologically could be liberated from the hypophysis Consequently ADH can have an inhibitory effect of gastric acid secretion in normal conditions

Obrink K J Bonedust meat mixture as a test meal for continous gastric secretion *Acta physiol scand* 1954 30 275

Verney E B The antidiuretic hormone and the factors which determine its release *Proc roy Soc B* 1947 135 25

Karlsson L. J Hirvonen Y Salorinne and K. Virtanen (Institute of Physiology University of Helsinki Finland) FUNCTIONAL STATUS OF THE HYPOTHALAMO HYPOPHYSEAL SYSTEM AFTER TWO WEEKS ADMINISTRATION OF ADRENALINE AND INSULIN IN RATS UNDER CONTINOUS INFLUENCE OF ETHANOL

Male rats divided in five groups were used in the experiment The first group received insulin (1 I U/100 g i m) and 10 % ethanol (3 % of weight via stomach tube) twice daily the second group insulin and water the third adrenaline (50 µg/100 g s c) and ethanol the fourth adrenaline and water and the fifth saline (0.5 ml s c) and water Blood alcohol con centrations and plasma electrolyte values were followed Sections of the neurosecretory system were stained with aldehydefuchsin for the neurosecretory material (NSM) and hematoxylineosine The amount of NSM was estimated microscopically and the mean volumes of nuclei and nucle oli in each group were calculated

Blood alcohol levels were around 1 pro mille two hours after ethanol ingestion In both insulin receiving groups sodium values were 5 per cent and chloride values 8 per cent higher while potassium values remained 5 per cent lower than those of the controls The supraoptic and para ventricular NSM diminished in the insulin water group but ethanol could subside this effect The neurohypophyseal NSM was scarcer in the insulin water group Simultaneous administration of insulin and alcohol was followed by enlargement of the supraoptic nuclei and shrinkage of the para venticular ones Diminution of nuclear volumes was observed after insulin In both adrenaline receiving groups there was plenty of NSM in both NSO and NPV Adrenaline and alkohol given simultaneously brought up a prominent nuclear enlargement in NSO and NPV

Thus it seems evident that adrenaline and insulin given simultaneously with ethanol enhance the functional activity of the neurosecretory system although these compounds separately all inhibit or have no influence upon it

Botting Regina M and Mary F Lockett Threshold effect of subcutaneous adrenaline noradrenaline and isoprenaline on water diuresis in rats *Arch int Physiol* 1961.69 36—45

Hirvonen J L Karlsson and K Virtanen Inhibition of the secretary function of the hypothalamo-hypophyseal system by ethanol in the rat *Ann Med exp Fenn* 1966 44

Verney E B The antidiuretic hormone and the factors which determine its release *Proc roy Soc B* 1947.135.25—106

Kjekshus J and B Bugge-Asperheim (Institute for Experimental Medical Research University of Oslo Ullevål Hospital Norway) THE OXYGEN CONSUMING EFFECT OF NORADRENALINE ON MYOCARDIUM DURING HAEMORRHAGIC HYPOTENSION IN DOGS

The oxygen consuming effect of Noradrenaline (NA) on the myocardial oxygen consumption (MVO_2) has been studied in intact nembutal anesthetized dogs bled to a reservoir and maintained for 3—5 hours at a constant aortic blood pressure (AP) ranging from 50—60 mm Hg

Myocardial blood flow has been estimated from hydrogen desaturation curves in the coronary sinus Hydrogen concentration was determined polarographically with a platinum electrode mounted on the tip of a cardiac catheter which was inserted under fluoroscopic control into the coronary sinus

Left ventricular pressure maximum rate of rise in the left ventricular pressure pulse (dp/dt) and cardiac output were measured

After bleeding MVO decreased initially to 50 % and then stabilized at 80 % of the control MVO_2 The cardiac external work (APxCO) decreased to less than 10 % of the control

Changes in MVO_2 were correlated to concomitant changes in dp/dt An even better correlation was obtained with the product of dp/dt and heart rate

NA was given intravenously with the reservoir closed External cardiac work increased insignificantly while mean left ventricular pressure rose 30 % Heart rate changed inconsistently MVO_2 increased more than 100 % associated with increase in the product of dp/dt and heart rate and in the product of left ventricular systolic pressure and heart rate

In order to dissociate between the pressor effect and the inotropic effect of NA a peripheral blocking agent diphenoxybenzamine was given In this group of experiments the rise in AP was prevented while the inotropic effect on the heart was unaffected There was only a minor increase in external work but almost unchanged increase in dp/dt Left ventricular systolic pressure increased to a much smaller extent

It is concluded that during haemorrhagic hypotension the product of dp/dt and heart rate is an important determinant of the myocardial oxygen consumption and that the calorigenic effect of NA can be explained solely by a more rapid rate of rise in left ventricular pressure pulse

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Klausen K. and B Rasmussen (Gymnastikteoretisk Laboratorium University of Copenhagen Denmark) ON THE LOCATION OF THE FRONTAL AXIS OF MOVEMENT IN THE LUMBAR PART OF THE HUMAN SPINE

The determination of the location of the frontal axis of movement between L_4 and L_5 is based on the observation that the long back muscles and the abdominal wall muscles *i e* m rectus abdominis are acting as antigravity muscles (Asmussen 1960 Klausen 1965) Integrated EMGs from these muscles were recorded simultaneously on a subject standing in two positions A The habitual symmetrical position with the hands crossed in front of the abdomen All subjects showed a slight to moderate activity only in the back muscles in this position B A slightly backwards leaning position with no activity in the back muscles and a slight to moderate activity in the rectus abdominis X ray pictures were taken of the subjects in positions A and B

The location of the line of gravity in the sagittal plane for the parts of the body above the level of L_4 was determined as described by Klausen (1965) The gravity line measurements were made with the subjects standing in positions A and B which were reproduced by means of the EMGs obtained simultaneously with the X ray pictures

From the X ray pictures and the gravity line measurements it could be shown that a shift of the activity from the back muscles to the rectus abdominis takes place when the line of gravity passes a point about 3 cm dorsal to the centre of the intervertebrate disc between L_4 and L_5 possibly indicating that the frontal axis of movement between these two vertebrae is located at the joints between the interarticular processes

Asmussen E The weight-carrying function of the human spine *Acta orthop scand* 1960 29 276—290
Klausen K The form and function of the loaded human spine *Acta physiol scand* 1965 65 176—190

Klinge E M J Mattila D Penttila and E Jukarainen (Department of Pharmacology University of Helsinki Finland) EFFECT OF PLASMA KININS ANGIOTENSIN AND VASOACTIVE AMINES ON PERFUSED HUMAN PLACENTA

Human placentas without umbilical cord were perfused *in vitro* using Locke s solution containing dextran The placental artery basal pressure was kept at 30—40 mmHg and its changes caused by injected bradykinin (20 μg) kallidin (20 μg) angiotensin (20 μg) 5HT (2—10 μg) histamine (10—15 μg) and adrenaline (500 μg) were recorded All these substances caused a rise in the perfusion pressure the effect of angiotensin being the most powerful one When calculated on the molar basis the equiactive doses were 1 for angiotensin 1.5 for 5HT 1 7 for bradykinin and kallidin 36 for histamine and about 200 for adrenaline In the same placenta the responses to repeated injections of bradykinin kallidin and angiotensin were very constant while different placentas showed great individual variations in their reactions

These vasoconstrictor responses were modified by several drugs added into the reservoir of the perfusion fluid Expressing concentrations of the drugs in μg/ml of the perfusion fluid the responses of the plasma kinins were partly inhibited by cinnarizine (0 1) indomethacine (10) hydrocortisone (50) and by phenylbutazone (40) The action of angiotensin was significantly enhanced by pethidine (25) and inhibited by cinnarizine (0 1) without being influenced by dibenzyline (0.2) The effect of 5HT was significantly inhibited by compound 48/80 (0 1) phenylbutazone (40) pethidine (25) cinnarizine (0 1) and by dibenzyline (0.2) The response of histamine was significantly inhibited by pethidine (25) dibenzyline (0.2) and by cinnarizine (0 1) the two first mentioned drugs even reversed the histamine response into vasodilatation Compound 48/80 did not alter the histamine effect The weak but long lasting effect of adrenaline was abolished by dibenzyline (0.2) but no reversal could be produced DCI (2.5) did not influence the effect of adrenaline

Knutsson E. and S Katz (Department of Physiology Karolinska Institutet Stockholm Sweden and Medical College of South Carolina USA)
STUDIES OF THE EFFECT OF ETHANOL ON THE IONIC PERMEABILITY OF MUSCLE FIBRE MEMBRANE

In a previous work ethanol was shown to reduce the resting potential and increase the membrane conductance of frog muscle fibres (Knutsson 1961) Since this conductance change may be due to alterations of the membrane permeability to one or several ions we have studied the effects of ethanol (2.3 g/l) on the membrane permeability to the diffusible ions present in high concentrations viz sodium potassium and chloride ions

The ethanol effect on the sodium permeability was studied by measuring membrane potential changes of fibres in external solutions with excess sodium chloride In these cases ethanol regularly caused a depolarization of the fibre membrane whereas the membrane potential was unaffected by ethanol in a number of different Na free solutions of the same tonicity The experiments strongly suggest that ethanol increases the membrane permeability to sodium ions

The effects on the permeability to potassium and chloride ions were studied by determining the influence of ethanol on the membrane conductance By using different external solutions the ions carrying current across the membrane were varied and thus information could be obtained concerning ethanol effects on the membrane permeability to a few selected ions From such experiments it was apparent that when the current across the membrane is carried mainly by potassium ions ethanol has no effect on the membrane conductance whereas when the current is carried also by chloride ions ethanol increases the membrane conductance The results obtained so far suggest that ethanol increases the membrane permeability to chloride ions but does not affect the potassium permeability of the membrane

Knutsson E *Acta physiol scand* 1961.52.242—253

XII Scandinavian Congress of Physiology Turku 1966
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Kohikko, A. (Cardiorespiratory Research Unit Department of Physiology University of Turku Finland) STUDIES ON THE HAEMODYNAMICS OF THE LAMB IN THE NEWBORN PERIOD

The cardiac output dye appearance time mean circulation time and central blood volume were calculated from the dye-dilution curves in lambs 1 to 10 days of age In addition the rectal temperature ECG respiratory rate and tidal volume were recorded These parameters were studied in normal condition in hypoxia and in hypercapnia Also the influences of epinephrine and THAM were tested

Hypoxia increased the heart rate if there was no acidosis In the first minutes the cardiac output increased slightly which did not correlate with the arterial oxygen saturation Later cardiac output returned to its initial level If hypoxia resulted in metabolic acidosis the cardiac output decreased below the initial level The respiratory rate was not significantly changed.

In hypercapnia the heart rate increased but returned to its initial level *in two to five minutes The effects on cardiac output depended on the* pH and the pCO_2 in the arterial blood Decreased pH resulted in a lower cardiac output but if pCO_2 was markedly elevated the cardiac output was *unchanged or elevated* The respiratory rate was elevated during the hypercapnia period

Epinephrine increased the cardiac output but this reaction was inhibited by metabolic acidosis

Infusion of THAM did not influence the circulation in the lamb in metabolic acidosis

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Kormano M (Department of Anatomy University of Helsinki Finland)
DEVELOPMENT OF THE TESTIS-RECTUM TEMPERATURE DIFFERENCE IN POSTNATAL RAT

According to a generally accepted conception vascular heat exchange mechanisms in the pampiniform plexus play the most important role in maintaining difference between rectal and testicular temperatures in mammalians this difference is about 3.5 C in the adult rat In this study the development of this temperature difference has been followed during the postnatal life period of the rat and its relationship to the onset of endocrine puberty development of scrotal sac and functional maturity of the seminiferous tubules has been evaluated

The intratesticular and rectal temperatures of 20 25 30 and 35 days old and adult rats were recorded The thermocouple needles were inserted during light ether anaesthesia while the recordings were carried out without anaesthesia The experimental procedure caused variations in the body temperature of the animals but did not significantly affect the testis-rectum temperature difference except in the adult group In this group the difference began to increase when rectal temperature rose over 37.6 C After external heating of the testis only the restoration of the normal temperature difference occurred within 10 minutes

The temperature gradient was approximately 3.5 C in the adult and 35 days old animals (Table) The 3 younger age groups (20 25 and 30 days) had significantly ($p<0.001$) higher intratesticular temperature The adult type of intratesticular temperature manifested clearly later than the endocrine puberty Although the continuous development of the scrotum may contribute to the result it seems probable that the vascular cooling mechanisms are mainly responsible for this change Angiographic observations were performed to correlate the development of the intratesticular vasculature in the postnatal rat to the testis rectum temperature difference

Table Testis-rectum temperature differences in postnatal rats

Age in days (No of animals)	Mean testis rectum difference (°C)	SD
Adult (9)	3.47	0.38
20 (4)	1.05	0.81
25 (4)	1.93	0.49
30 (4)	2.04	0.56
35 (5)	3.33	0.79

Kormano M and M Niemi (Department of Anatomy University of Turku Finland) CONTRACTILITY OF THE SEMINIFEROUS TUBULES OF THE RAT TESTIS

Seminiferous tubules of both adult and postnatal prepuberal rat testis were explanted in warm Tyrode s solution and their contractions registered by cinematography in transmitted light medium power microscope objectives were used The adult tubules contracted irregularly with a period of about 10 sec and an amplitude of about 15 μ Oxytocin in a concentration of $2x10^{-4}$ I U increased the frequency of the contractions During the prepuberal period the first spontaneous contractions were observed in 14 days old animals The contractility was already then sensitive to oxytocin Histological observations on the boundary tissue of the seminiferous tubules showed that the basement membrane contained contractile cells during the whole postnatal period There were no morphological alterations visible at the light microscope level at the time of the appearance of the first spontaneous contractions

Kumento, A and E Kulonen (Department of Medical Chemistry University of Turku Finland) REFLECTION OF COLLAGEN SYNTHESIS IN EXPERIMENTAL GRANULATION TISSUE ON THE PLASMA PROTEIN BOUND HYDROXYPROLINE

Experimental granulomas were induced by implantation of viscose cellulose sponges (four pieces 20x10x10 mm each) subcutaneously in the backs of adult rats The animals were killed at intervals from [illegible] to 40 days at the age of 3 months The blood was collected in heparinized tubes and the plasma separated The proteins were precipitated with ethanol (final concentration [illegible] % v/v) and hydrolyzed in saturated barium hydroxide solution for 16 hrs at +130 C in sealed tubes The liberated hydroxyproline was determined according to Le Roy *et al* (1964) The basal value was about 2.5 μg/ml but during the period from the 10th to the 20th days after the implantation there was a significant rise The peak (4.1 μg/ml) was on the 15th day These data are in good agreement with that period when slices of granulomas are capable of synthetizing collagen (Supported by Sigrid Jusélius Foundation)

Le Roy Kaplan Udenfriend and Sjoerdsma *J biol Chem* 1964.239.3350

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Kärkkäinen J A Lehtonen and E Haahti (Department of Medical Chemistry University of Turku Finland) DETERMINATION OF MONOSACCHARIDE COMPONENTS OF MUCOPOLYSACCHARIDES BY GAS CHROMATOGRAPHY

Gas chromatography of sugars as trimethylsilyl ethers (Sweeley *et al* 1963) offers a convenient way for the determination of monosaccharide components of mucopolysaccharides The ratio of glucosamine to galactosamine as well as their absolute amounts in a mucopolysaccharide mixture can be rapidly and quantitatively analysed after complete hydrolysis (Karkkainen *et al* 1965) Iduronic and glucuronic acids can be *separately analysed as lactones after controlled acid hydrolysis* Neutral sugars occurring in the hydrolysates can also be identified and quantitatively determined as trimethylsilyl ethers

Because of the complexity of natural mucopolysaccharide mixtures gas chromatography of the monosaccharides is seldom possible without prefractionation of the hydrolysate Ion exchange procedures using aqueous solvents can be employed for purification of amino sugars Silicic acid column chromatography of the monosaccharides as trimethylsilyl ethers employing non polar solvents seems however more suitable for group separation of amino sugars uronic acids and neutral sugars Using ion exchange prefractionation the glucosamine and galactosamine contents of the mucopolysaccharides of aorta skin laryngeal cartilage and umbilical cord have been determined with gas chromatography The same method has been applied for the analysis of hexosamines and neutral sugars of the epithelial mucin of hagfish *Myxine glutinosa* (Lehtonen *et al* 1966b) Silicic acid column prefractionation has been used in the analysis of mucopolysaccharides of skin and aorta Due to the sensitivity of the gas chromatographic methods it was possible to analyse the monosaccharide components of mucopolysaccharides isolated from cellulose acetate electrophoresis sheets (Lehtonen *et al* 1966a)

Karkkainen J A Lehtonen and T Nikkari *Determination of glucosamine and galactosamine by gas chromatography J Chromatog* 1965 20 457—462

Lehtonen A J Karkkainen and E Haahti Gas chromatographic characterization of the electrophoretically separated fractions of acid *mucopolysaccharides J Chromatog 1966a* In press

Lehtonen A J Karkkainen and E Haahti Carbohydrate components in the epithelial mucin of hagfish Myxine glutinosa *Acta chem scand* 1966b In press

Sweeley C C R Bentley M Makita and W W Wells Gas liquid *chromatography of trimethylsilyl derivatives of sugars and related* substances *J Amer chem Soc* 1963 85 2497—2507

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Landgren S., H Silfvenius and D Wolsk (Department of Physiology University of Goteborg Sweden) THE SENSORY INPUTS TO THE SECOND CORTICAL PROJECTION AREA OF THE GROUP I MUSCLE AFFERENTS

The Group I muscle afferents from the contralateral forelimb of the cat project to the postcruciate dimple (Pcd) of the sensori motor cortex (Oscarsson and Rosén 1963) and to the lower lip of the cortical fold formed by the anterior suprasylvian sulcus (Landgren and Wolsk 1966) In this investigation the sensory projections to the suprasylvian fold were studied

The experiments were made on cats anaesthetized with chloralose Muscle skin and joint nerves from contra and ipsilateral fore and hindlimbs were stimulated electrically with graded strength The afferent volley was monitored from the nerve trunks The vestibular nerves were prepared for electrical stimulation and auditory stimuli were presented The focal potentials evoked by these stimuli were recorded along systematically placed microelectrode tracks through the suprasylvian fold

The Group I nucleus in the lower lip has a mediolateral extent of about 1 mm and a rostro-caudal extent of 1—2 mm Short latency responses were evoked in this region by low threshold skin afferents (7 msec) joint afferents (10—14 msec) and high threshold muscle afferents from the contralateral forelimb Ipsilateral vestibular responses (4—7 msec) and click responses were secorded in the caudal part of the nucleus Low threshold skin and high threshold muscle afferents from the contralateral hindlimb and from the ipsilateral limbs evoked small and rather late responses in the Group I nucleus Electrical stimulation of Pcd evoked a large focal potential in the Group I nucleus of the suprasylvian fold

An interesting organization was found in the upper lip of the fold Low threshold skin and high threshold joint and muscle afferents from the contralateral forelimb projected to the rostral and lateral parts of the upper lip The same types of afferents from the hindlimb evoked responses medially and caudally In one case a response to stimulation of Group I muscle afferents from the contralateral gastrocnemius muscle and in another case a response to low threshold knee joint afferents were recorded there

Oscarsson O and I Rosén *J Physiol (Lond)* 1963 *169* 924—945
Landgren S and D Wolsk *Life Sciences* 1966 *5* 75—79

Lang H and I Lehtinen (Department of Physiology University of Turku Finland) SWEATING AND THE GALVANIC SKIN REACTION TWO INDEPENDENT FUNCTIONS OF THE SWEAT GLANDS?

The effect of the arterial occlusion upon sweating and the galvanic skin reaction GSR has been studied in cats The sudomotoric activity was induced by electrical stimulation of the distal stump of the sectioned sciatic nerve The GSR was recorded as a potential change from the surface of the foot or toe pads with metal macroelectrodes As an indication for sweating a semiquantitative dye reaction was used first adapted for this purpose by Silverman and Powell (1944)

Occlusion of the arterial circulation to the leg had a different effect upon sweating and the GSR the sweating disappeared often before any prominent change in the GSR could be observed The recovery of sweating and the GSR occurred also unsynchronously after releasing the circulation

On the basis of these and some other observations it is postulated that the secretory and the bioelectrical activity recorded from the skin surface represent two different and partially independent functions of the sweat glands

Silverman J J and V E Powell Studies on palmar sweating I A technique for study of palmar sweating *Amer J med Sci* 1944 208 297—299

Larsen J A. (Department of Physiology University of Aarhus Denmark)
THE EFFECT OF GLYCINE AND OXYGEN BREATHING ON THE ELIMINATION RATE OF GLYCEROL AND ETHANOL IN CATS

The breathing of oxygen was found to decrease the elimination rate of glycerol from a mean value of 52 7 to 36.5 uMol/kg/min whereas the elimination rate of ethanol was unaffected In experiments in which the elimination rate of glycerol was comparatively low no such effect of oxygen could be demonstrated The elimination rate of both glycerol and ethanol could be increased by infusion of glycine ½ g/kg bodyweight This effect of glycine could be almost completely abolished by oxygen breathing *Thus the elimination rate of glycerol was increased from 35 6* to 57 4 uMol/kg/min by infusion of glycine and was reduced to 36 1 uMol/kg/min during oxygen breathing The elimination rate of ethanol was increased from [illegible]9—41 8 uMol/kg/min by infusion of glycine and was reduced to 33 8 uMol/kg/min during oxygen breathing

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Larsen V (Dumex Ltd Copenhagen Denmark) THE BIOASSAY OF THYROID BY O-CONSUMPTION IN MICE

According to the literature there is no fixed relation between the content of iodine in desiccated thyroid and the increase of the metabolic rate in human beings and animals caused by the latter this renders a biological assay desirable It would seem logical to base a bioassay on the ability of the gland to increase the metabolic rate as it is just this effect that is utilized clinically

A) method In the method to be described here 3 groups each comprising 24 mice are employed for practical reasons The first group is given a small dose of the test substance e g 0 6 mg desiccated thyroid per mouse per day the second group a moderate dose e g 1.2 mg Standard Thyreoidea (Dumex Standard Thyreoidea 1963) and the third group a large dose e g 2 4 mg test thyroid The metabolic rate in the three groups is measured for 22—25 days and the increase is expressed by the size of 3 areas (area between a horizontal base and the 3 curves) The relative potency of the test substance is calculated by interpolation on the presumption that the curve obtained with the standard group is situated between the two other curves

B) the exactness of the method has been examined 1) by performing measurements on 3 groups of animals which are given 3 different doses of *Standard Thyreoidea* on the lines of the description given above By regarding the moderate dose as standard substance and the large respectively small dose as test substance the relative potency of the imaginary test substance can be found as mentioned by interpolation by which the size of the error is directly shown By employing a dose-effect curve for Dumex Standard Thyreoidea 1963 the 5 other percental errors that would have resulted from the 5 other possible placings of the 3 groups of mice can be calculated with satisfactory exactness 2) by performing measurements on 3 groups of mice receiving the same dose of *Standard Thyreoidea* per mouse per day for 22—25 days Each of these groups can here be considered as the standard group and the 2 others as test substance groups By calculating as above the potency of the imaginary test substance in the 3 cases the 3 possible errors resulting from the different placings of the animal groups are found

10 bioassays of the two above mentioned types resulted in 40 possible results calculated or found by experiment the simple mean deviation from the group acting as standard was approx 11 % and the standard deviation (S D) was approx 16 %

Lennerstrand G and U Thoden (Nobel Institute for Neurophysiology Karolinska Institutet Stockholm Sweden) DYNAMIC ANALYSIS OF PRIMARY AND SECONDARY MUSCLE SPINDLE ENDINGS WITH AND WITHOUT STIMULATION OF SINGLE FUSIMOTOR FIBRES

The responses of primary and secondary muscle spindle afferents were studied during length changes of the ankle extensors of the cat Periodically recurring streches and relaxations with triangular sinusoidal or parabolic length time relations were used having periods from 32 to 1/2 seconds They were imposed on de-efferented spindles as well as on spindles activated by stimulation of single dynamic on static fusimotor fibres The instantaneous impulse frequency of the ending was displayed on the y axis and muscle length on the x axis of a CRT oscilloscope to form impulse frequency length loop diagrams

As the ultimate aim of the investigation is analogous stimulation of the muscle spindle rather extensive measurements from the loop diagrams of position and velocity sensitivity have been performed The possibility that sensitivity to acceleration might also be involved in the response has been ruled out by observing the reaction to parabolic length changes Typical loop diagrams will be shown and the information that can be extracted from them explained

Some of the most interesting features revealed by the investigation concern the effect of static fusimotor stimulation on the endings Two types of responses were found Both types of responses consisted of an increased firing under static conditions and of an unchanged velocity sensitivity compared to the unstimulated ending The differences were in the effect of the stimulation on position sensitivity In the first type which was most common in primary endings position sensitivity did not change while in the second type which was rather scarce in primaries position sensitivity was increased

The secondary endings which are known to be innervated almost exclusively by static fibres predominantly showed the above second type of response on fusimotor stimulation

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Liljestrand G (Department of Pharmacology Karolinska Institutet Stockholm Sweden) DISTRIBUTION OF THE PULMONARY BLOOD FLOW

The observation that low O_2-as well as high CO_2-tension in the alveolar air causes a rise in pulmonary artery pressure (PAP) led to the hypothesis that this is a consequence of a normal mechanism by which the distribution of the pulmonary blood flow is directed from poorly ventilated areas thus bringing about favourable conditions for complete oxygenation of the blood (von Euler and Liljestrand 1946)

In 1958 I was able to demonstrate on the isolated and perfused cat lungs that ventilation with gas mixtures of low O_2-or high CO_2-tensions usually led to increased PAP and that this effect was closely correlated with a lowering of the pH Low O_2-tension was found to call forth a production of lactic acid in the lungs (Liljestrand 1958)

Two years later Fishman Fritts and Cournand (1960) observed that patients inhaling 3—5 % CO_2 in air displayed no increased PAP Since this was in opposition to my explanation of the regulatory mechanism the question was reinvestigated by Bjurstedt Matell and myself (1961) Using anesthetized and curarized dogs with constant artificial respiration we could easily obtain the expected rise in PAP during low O_2-tension as well as during infusions of lactic or hydrochloric acids whereas bicarbonate infusion caused a lowering of PAP The production of lactic acid in the hypoxic lungs was demonstrated indirectly as well as directly The result by Fishman *et al* was due to too low a concentration of CO_2 during spontaneous respiration with overventilation as a consequence of the extra stimulation

In 1962 Bergofsky Lehr and Fishman obtained similar results as we had found — though they did not quote our paper of 1961 In his review in the Handbook of Physiology (1963) Fishman acknowledged (1963) the general importance of our hypothesis but did not take a definite position regarding the underlying mechanism and somewhat later (1964) the American group (Enson *et al* 1964) came to the conclusion that a rise in blood pH was accompanied with a fall or with no change in PAP despite increased blood flow They also found that PAP was correlated with both oxygen saturation and hydrogen ion concentration to a higher degree than with either of these two variables considered singly

Quite recently the situation has changed somewhat In the end of January this year I spent by invitation a week in New York where a kind of symposia had been arranged by Cournand and Fritts at the Bellevue Hospital and by Fishman at the Medical Centre of Columbia University Among the many communications given and discussed one was of very special interest in this connection It had been possible to produce in the rabbit reversible infiltrations in patches within the lungs and in such rabbits lowering of the O_2 tension caused the production of lactic acid thus illustrating the power of the lung to evoke lactic acid locally This observation strengthens the view expressed above

The effects of reduced O_2 and increased CO_2-tension are not only of importance as a local mechanism but may also play a role for certain pathological states especially for the development of Cor Pulmonale when the effect is exercised on the lungs as a whole thereby putting a great strain on the right ventricle

von Euler U S and G Liljestrand *Acta physiol scand* 1946 12 301
Liljestrand G *Acta physiol scand* 1958 44 216
Fishman A P H W Fritts Jr and A Cournand *Circulation* 1960 22 220
Bjurstedt H G Liljestrand and G Matell *Ciba Foundation Study Group* No 8 1961 63
Bergofsky E H E Lehr and A P Fishman J clin Invest 1962 41 1492
Fishman A P in *Handbook of Physiology — Circulation* II 1963 1667

Lindstrom L and B J Meyerson (Department of Pharmacology University of Uppsala Sweden) EFFECTS OF CHOLINERGIC AND ANTI CHOLINERGIC AGENTS ON ESTROGEN PROGESTERONE ACTIVATED ESTRUS BEHAVIOUR IN OVARIECTOMIZED RATS

Estrus behaviour in female rats is dependent on ovarian hormones In ovariectomised rats sexual receptivity can be restored by treatment with estrogen followed after a certain time interval by progesterone (Beach 1942) There is strong evidence that these hormones act directly on structures within the hypothalamus (Harris *et al* 1958 Lisk 1962)

A possible antagonistic relation between cerebral monoamines and hormone activated estrus behaviour in the rat was recently demonstrated (Meyerson 1964)

In the present investigation drugs which act on central nervous cholinergic functions were studied with regard to their effect on estrogen progesterone activated estrus behaviour

Ovariectomised Sprague Dawley rats 250—300 g were treated with estradiol benzoate 10 μg/kg and 48 hours later 0 4 mg/animal progesterone The observations of estrus behaviour were performed 4—9 hours after the progesterone injection

Pilocarpine (25 mg/kg) significantly decreased the heat response its effect being inhibited by atropine (1 mg/kg) but not by metylatropine (5 mg/kg) Arecoline (5 mg/kg) showed a short-acting estrus inhibitory effect that lasted for about half an hour Tremorine however which reportedly increases the central cholinergic tone (Everett 1956) did not reduce the heat response

The present results indicate that besides the central monoaminergic mechanisms demonstrated earlier a possible connection also exists between hormone induced estrus behaviour and central nervous cholinergic functions

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Everett G M L E Blockus and J M Sheppard *Science* 1956 *124* 79
Harris G W R P Michael and P P Scott *Ciba Found Symp on the Neurological Basis of Behaviour* 1958
Lisk R D *Amer J Physiol* 1962.*203* 493
Meyerson B J *Acta physiol scand.* 1964.*63* Suppl 241

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Louhija A (Wihuri Research Institute Helsinki Finland) LIPID METABOLISM IN SEVERE EXPERIMENTAL HAEMORRHAGIC ANAEMIA

Severe bleeding causes a marked hyperlipidaemia (Boggs and Morris 1909) The 'haemorrhagic lipaemia' is due to the loss of red cells while the loss of plasma is of little significance (Starup 1937) The most probable aetiological factor is anaemic hypoxia

The exact mechanism of haemorrhagic lipaemia is not known Some pertinent aspects have been investigated recently (Louhija 1965 Louhija and Hirvisalo) *It was observed that serum FFA and glycerol concentrations fell during the development of haemorrhagic lipaemia* No increase in the production of FFA occurred when adipose tissue slices of bled rats were incubated *in vitro* as compared with sham bled control rats Thus haemorrhagic lipaemia obviously cannot be a result of increased peripheral lipid mobilization This is further corroborated by the fact that the turnover of liver fatty acids derived from the plasma FFA pool seemed not to be accelerated Thus no ketosis occurred during the development of haemorrhagic lipaemia and the uptake and turnover in the liver of intravenously injected albumin bound 1 ^{14}C-palmitic acid was not increased Neither seems the synthesis of fatty acids *de novo* to be increased as it was observed that the *in vivo* incorporation of intraperitoneally injected acetate 1 ^{14}C *into liver and serum lipids was decreased in haemorrhagic* lipaemia Haemorrhagic lipaemia thus seems to be neither due to increased peripheral lipid mobilization nor to increased synthesis of fats *de novo* It is therefore obvious that the lipaemia is due to retarded elimination of the lipids from the circulation

Louhija A *Ann Med exper Fenn* 1965 43 Suppl 2
Louhija A and Hirvisalo to be published
Starup Undersogelser over experimentel hyperlipaemi Copenhagen 1937

Lundborg P (Department of Pharmacology University of Goteborg Sweden) SOME OBSERVATIONS ON THE FUNCTION OF AMINE STORAGE GRANULES

The uptake of H metaraminol by the adrenal medullary granules *in vitro* has been studied Metaraminol is not taken up by the ATP Mg++-dependent uptake mechanism However by an analysis according to the method of Lineweaver and Burk it is shown that metaraminol is a competitive inhibitor of the ATP Mg++-dependent uptake of adrenaline Reserpine even in rather high concentration has no influence on the accumulation of metaraminol in the granules It is shown that there is a good correlation between the uptake of metaraminol and the concomitant release of adrenaline and noradrenaline from the granules from which might be suggested that metaraminol is taken up by displacing the amines from their storage sites By an indirect approach it is shown that the uptake of metaraminol is not stereospecific but has preference for the (—) form

Dopamine is taken up by the granules not only by the ATP Mg++ -dependent uptake mechanism but also by another mechanism in the same way as metaraminol

Thus there seem to exist two uptake mechanisms in the amine granules *i e* one ATP Mg++-dependent mechanism sensitive to reserpine and the other acting by displacement of endogenous amines Dopamine is capable of utilizing both and metaraminol only the latter mechanism

Lundin J (Department 1 Research Institute of National Defence Sundbyberg Sweden) DIFFERENT FISH MUSCLE CHOLINESTERASES AND THEIR PROPERTIES

At least three different kinds of cholinesterases in body muscle tissues from fish have been described One is a typical acetylcholinesterase acetyl choline hydrolase E C 3 1 1 7 not splitting butyrylcholine It is confined to the nerve muscle junctions and easily extracted The properties of this enzyme in glodfish *Carassius auratus* have been described (Lundin 1958) From body muscle extracts of the dwarf sheat fish *Amiurus nebulosus* Kover Szabolcs and Csabai (1964) prepared a myosin fraction containing cholinesterase activity but this enzyme splits butyrylcholine and is localized intracellularly in muscle cells (Kovacs *et al* 1961) Lundin (1960) demonstrated another butyrylcholine splitting cholinesterase in the body muscle tissues of many species of salt water fish Investigations on plaice *Pleuronectes platessa* have indicated that this cholinesterase is located in the muscle cell wall The binding to cell structures made it impossible to purify the enzyme by conventional methods However by autolysis involving the action of a certain bateria strain (*Cytophaga sp*) the enzyme may be solubilized (Lundin 1964 1966) and has now been purified about 20 000 times

The properties of different cholinesterases present in fishes and their possible physiological functions will be discussed

Lundvall J., S Mellander and H Sparks (Department of Physiology University of Goteborg Sweden) MYOGENIC RESPONSE OF PRECAPILLARY SPHINCTERS IN EXERCISING SKELETAL MUSCLE

Previous work from this laboratory demonstrates that high transmural pressure causes myogenic constriction of precapillary sphincters resulting in decreased capillary surface area available for exchange and this may be important in preventing edema formation Conversely muscular exercise causes relaxation of precapillary sphincters If the latter is the dominating influence during work in the presence of increased hydrostatic load gross edema could result The present study evaluates the relationship between these two competing factors

Capillary filtration coefficient (CFC) reflecting precapillary sphincter tone and blood flow of cat calf muscle were observed at various trans mural pressures Muscle work was produced by stimulation of the severed sciatic nerve using parameters which do not excite sympathetic fibres (2V 0 1 msec 1/sec)

Increasing transmural pressure 20 to 30 mm Hg above normal caused a 30 % decrease in CFC of resting muscle Passive distention of the resistance vessels was closely balanced by increased active tone

At normal transmural pressure exercise reduced flow resistance to 1/3 of the resting value and CFC increased approximately twofold Resistance fell even more when transmural pressure was increased during work supporting the view that exercise opposes myogenic autoregulation of resistance vessels In contrast high transmural pressure decreased CFC approximately 50 % This demonstrates the persistance of the myogenic response of precapillary sphincters to increased transmural pressure in the face of increased metabolic demand Perfusion of the exercising calf muscle with cooled blood (33°C) abolished this response of the precapillary sphincters This suggests that the observed effect of transmural pressure on CFC is related to active changes in vascular tone

Maintainance of precapillary sphincter tone in spite of increased metabolic demand may be one important factor preventing gross edema formation in the legs of man during exercise

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Lomo T (Laboratory of Neurophysiology Institute of Anatomy University of Oslo Norway) FREQUENCY POTENTIATION OF EXCITATORY SYNAPTIC ACTIVITY IN THE DENTATE AREA OF THE HIPPOCAMPAL FORMATION

Earlier studies on the hippocampus have shown that repetitive stimulation of afferent pathways leads to a marked increase of spike generation However the responsible mechanism is largely unknown.

Extracellular responses of dentate granule cells evoked by repetitive stimulation of the entorhinal area or perforant path fibres were recorded simultaneously with two microelectrodes one electrode recording from the layer of perforant path synapses on the granule cell dendrites the other from the layer of granule cell bodies

After an initial depression lasting for a few seconds repetitive stimulation led to a large potentiated response compared to the response evoked by a single volley This effect frequency potentiation was seen as an increase of the amplitude and a decrease of the latency of the population spike and as an increase of the rate of rise and amplitude of the extracellular excitatory synaptic potentials The most effective stimulation frequency was 12 to 15 c/sec No potentiation was seen with frequencies lower than 6 or higher than 50 c/sec Presumably the frequency potentiation is partly due to an increased output of excitatory transmitter The process may serve as a method for control of nervous activity

When trains of stimuli (12 c/sec 10 sec duration) were repeated with intervals of rest of 5—10 min duration the potentiated responses evoked by the later train were larger appeared earlier in the train and had shorter spike latencies than the responses evoked by the first train of the series This represents an example of a plastic change in a neuronal chain expressing itself as a long lasting increase of the synaptic efficiency The effect which may last for hours is dependent upon repeated use of the system.

Supported by a US Public Health Service Research Grant NB 04764 from the National Institute for Neurological Diseases and Blindness which is gratefully acknowledged.

Loyning Y (Institute of Physiology University of Oslo Norway)
PRESYNAPTIC EFFECT OF BARBITURATE ON NEUROMUSCULAR TRANSMISSION

The powerful neuromuscular blocking action of barbiturates has been thought to be due mainly to a postsynaptic effect (see Thesleff and Quastel 1965) However biological assays have shown that increasing concentrations of barbiturates reduce and eventually block the release of acetylcholine at the neuromuscular junctions (Straughan 1961 Matthews and Quilliam 1964) In the present study the effect of pentobarbitone sodium (Nembutal®) in concentrations of from 25 to 300 mg/l (1—12 $\times 10^{-4}$ M) has been examined Isolated rat diaphragm phrenic nerve preparations were used either as unblocked preparations or as Mg paralysed or high Mg low-Ca paralysed preparations

In unblocked preparations exposed to 300 mg/l of Nembutal® contractions sometimes began to disappear after 5 min exposure and were usually abolished after about half an hour With 100 mg/l of Nembutal® contractions sometimes began to disappear after 5 min exposure and were usually abolished after about half an hour With 100 mg/l of Nembutal® contractions could persist for more than an hour Intracellular recording from unblocked partly contracting preparations exposed to 100—200 mg/l of Nembutal® revealed a transition of action potentials into decaying EPPs which suddenly disappeared The MEPPs decreased concomitantly Intracellular recordings from paralysed preparations revealed that the EPP amplitudes decreased more than MEPP amplitudes during Nembutal® application (100—200 mg/l) the extra decrease could be accounted for by a decrease in quantal content It is concluded that both pre and postsynaptic effects of barbiturates contribute to the impairment of neuromuscular transmission The mechanism by which barbiturates reduce transmitter output could be the same as that suggested for the effects on afferent nerve terminals in the spinal cord (Loyning et al 1964)

Loyning Y T Oshima and T Yokota *J Neurophysiol* 1964 *27* 408—428
Matthews E K and J P Quilliam *Brit J Pharmacol* 1964 *22* 415—440
Straughan D W *J Pharm Pharmacol* 1961 *13* 49—52
Thesleff S and D M J Quastel *Ann Rev Pharmacol* 1965 *5* 263—284

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Malmfors, T (Department of Histology Karolinska Institutet Stockholm Sweden) HISTOCHEMICAL STUDIES ON THE RELEASE OF CATECHOLAMINES FROM THE ADRENERGIC NERVES PRODUCED BY NERVE IMPULSES

Studies on the adrenergic nerves in rat iris with the fluorescence histochemical method of Falck and Hillarp have given a great deal of very important information about the mechanisms of the adrenergic nerves (see Malmfors 1965) In particular the ability of the adrenergic nerves to take up exogenous noradrenaline (NA) has been clarified in a direct manner But also the release of the adrenergic transmitter has been studied (Malmfors 1965) Here some further studies on the release by nerve impulses will be reported It has been found that the transmitter is released in all probability from the varicosities along the adrenergic terminals and that the amine granules play an important role in the release Reserpine and inhibitors of the NA synthesis have been used with good results in combination with stimulation They accelerate the release of the transmitter the latter due to the fact that the resynthesis like the recapture of the released NA is important in keeping a constant transmitter level The release of the transmitter is inhibited by adrenergic neuron blockers like guanethidine and bretylium While exogenous NA taken up into the adrenergic nerves after depletion of the endogenous NA with reserpine cannot be released by nerve impulses the catecholamine present after injection of dopamine (DA) preceded by nialamide to a reserpinized animal can be released This indicates that the uptake and accumulation of DA is somewhat different from that of NA

Malmfors T Studies on adrenergic nerves The use of rat and mouse iris for direct observations on their physiology and pharmacology at cellular and subcellular levels *Acta physiol scand* 1965 64 Suppl 248 1—93

Manninen K and A Pekkarinen (Department of Pharmacology University of Turku Finland) EFFECT OF DRUGS ON THE URINARY ADRENALINE SECRETION BLOOD GLUCOSE AND BODY TEMPERATURE DURING INSULIN SHOCK OF RATS

The blood glucose content of control rats 0 88 g/l decreased within 3 hrs to 0.24 g/l after 4 U/kg i.m and increased in 7 hrs to 0 60 g/l Administration of neuroleptic thymoleptic or psychosedative drugs (thioridazine chlor prothixene chlorpromazine chlopenthixol propipendyl promethazine imipramin chlordiazepoxide 30 10 25 10 20 25 25 40 mg/kg respectively i m) prevented or diminished the hypoglycaemic effect of insulin clearly The lowest mean blood glucose values were then between 0.57—0 87 g/l except for protipendyl and promethazine 0 45 and 0.54 g/l respectively The mean hypoglycaemic response was of shorter duration than in the insulin control group These drugs inhibited or decreased also the adrenaline secretion in insulin shock of rats effectively and promethazine and chlor diazepoxide moderately (Pekkarinen Manninen and Maenpaa 1965) When injected without insulin these drugs had a slight hyperglycaemic effect

After P 286 opipramol oxypertine trifluproniazine perphenazine or pericyazine (10 20 20 10 2.5 mg/kg i m) the hypoglycaemic response to insulin was only slightly weaker than in the insulin control group Opipramol is an effective P 286 oxypertine and pericyazine moderately effective inhibitor of adrenaline secretion in insulin shock while trifluproniazine and perphenazine have not been effective

Soventol®(25 mg/kg) did not inhibit the hypoglycaemic response of insulin although it is effective inhibitor of adrenaline secretion in insulin shock Its inhibitory effect upon the adrenomedullary secretion is more specific than the inhibition of hypoglycaemia Also chlorpromazine and chlorprothixene have shown the specific inhibition of adrenomedullary secretion in haemorrhagic shock (Pekkarinen Manninen and Thomasson 1966) indicating that the effect of several neuroleptic drug is more specific than the inhibition of hypoglycaemia

47 new drugs were studied for their inhibiting effect on the adrenaline secretion in insulin shock in addition to our earlier observations of neuroleptic and psychosedative drugs Among new neuroleptics psychosedatives psychotonics antiepileptics muscle relaxants anti parkinsonian drugs antihistaminics antitussives antihypertensives sympatholytic and ganglionic blocking drugs only few had a clear inhibiting effect upon adrenomedullary secretion in insulin shock

Oxypertine chlorprothixene chlorpromazine chlopenthixol pericyazine and triflupromazine were hypothermic after insulin (the lowest mean temperatures between 34 1 and 30 4 C°) Short hypoglycaemia appeared already in one hour and hypothermia was slow with minimum in 4—5 hrs

Pekkarinen A K Manninen and M Maenpaa *Acta endocr (Kbh.)* 1965.50 Suppl 100 141

Pekkarinen A K Manninen and M Maenpaa *Mechanisms of Relese of Biogenic Amines* Wennergren Centr Internat Sympos Series vol 5 Stockholm Ed by v Euler Rosell and Uvnas Pergamon Press Oxford 1965 261—266

Markkanen T and V Nanto (Medical Clinic and Department of Medical Chemistry University of Turku Finland) *CALCIUM PHOSPHORUS BALANCE AND ETHANOL*

Oral or intravenous administration of ethanol is followed by an increase in the urinary elimination of calcium (*e g* Kalbfleisch *et al* 1961) We have investigated the effect of intravenous infusion of ethanol (50 ml of 94 per cent ethanol in 500 ml of physiological saline infused during a period of three hours) on the levels of calcium and inorganic phosphorus in serum and urine of hospital patients None of test persons (14 women and 2 men) was an alcoholic

In most cases an elevation of serum calcium (mean increase of 16 8 per cent at 2 hours after the beginning of the infusion) and inorganic phosphorus (29 7 per cent respectively) was observed The changes were statistically significant Urinary elimination of calcium and especially inorganic phosphorus (in relation to the excreted creatinine) increased already during the infusion and was greatest at the time when the elevated values in serum were decreasing after the end of infusion In two test persons no change was observed in calcium and phosphorus concentrations of serum during infusion of ethanol

The data suggest that ethanol even in small doses (less than 1 mg per gm of body weigth) is capable of producing changes in the equilibrium of calcium and inorganic phosphorus in blood The mechanism of these changes is obscure for the present

Kalbfleisch J M R H Lindeman and W O Smith *J Lab clin Med* 1961 *58* 833

Marsden N V B and A M M Zade-Oppen (Institute of Physiology Uppsala Sweden) JET EXPULSION OF HAEMOGLOBIN DURING PHOTODYNAMIC HAEMOLYSIS

Haemolysis was studied by phase and interference contrast cinemicrography While some haemolytic agents destroyed the cells (e g extreme pH values) haemolysis produced by other systems e g hypotonic and posthypertonic systems and many lysins was usually manifest only as a change in the optical contrast of the cells However in photodynamic haemolysis of cells sensitized with Rose Bengal part at least of the haemoglobin was released in a jet Haemolysis appeared to be initiated by jet expulsion and while most often single up to five jets were observed emerging from one cell It is of course problematical whether the more usual apparently jet less haemoglobin escape is truly jet less or consists of many small jets which cannot be resolved by the optical system

The visible front of the jet travelled about 5 μ in 1/50 second (250 μ/sec) but this represents a minimal velocity as there is uncertainty both as regards the direction and true position of the jet front

A jet may either propel the cell in the opposite direction or move a second cell if it impinges on it This suggests a bulk outflow of matter (presumably largely haemoglobin and water) a conclusion which also is supported by the fan or mushroom like shape of the jet cloud with its narrowest point at the cell surface If this interpretation is correct part at least of the haemoglobin escape in photodynamic haemolysis is due to a bulk non-diffusive flow The apparent integrity of the post haemolytic ghost suggests that the defect through which bulk flow occurs can be resealed

These studies were made at the Department of Biophysics King s College London in collaboration with Dr H G Davies

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Matell G and B Wigertz (Laboratories of Aviation and Naval Medicine Department of Physiology Karolinska Institutet Stockholm Sweden)
EFFECTS OF AMOBARBITAL METHOCARBAMOL AND CHLOR-PROMAZINE ON THE INITIAL PHASE OF EXERCISE HYPERPNEA.

Ventilatory responses to work periods of 20-sec (50 200 400 600 and 800 kpm/min) in the recumbent position (bicycle ergometer) have been studied in six healthy male subjects during (*A*) control condition and following administration of (*B*) amobarbital (3 mg/kg body weight) (*C*) methocarbamol (38 mg/kg body weight) and (*D*) chlorpromazine (0.8 mg/kg body weight) In series *A* *B* and *C* the rise in pulmonary ventilation during the first 20 sec of exercise was approximately of the same magnitude at corresponding work loads and varied between 4.7 and 7.2 l/min In series *D* the initial rise of ventilation in response to exercise was significantly less (14—26 %) than in the control condition The ventilatory response to inhaled CO_2 was not affected by the drug administration It was concluded that chlorpromazine has a depressant action on the initial phase of exercise hyperpnea which is not mediated by an influence on chemosensitive structures but rather on other parts of the nervous system supposedly the γ-system

McReynolds J S* D Ottoson and G M Shepherd** (Department of Physiology Karolinska Institutet Stockholm Sweden) SENSITIVITY CHANGES IN ISOLATED FROG MUSCLE SPINDLE DURING AND AFTER STRETCHING

Previous work (Ottoson and Shepherd 1965 Shepherd and Ottoson 1965) has shown that the impulse discharge and the underlying receptor potential of the frog s muscle spindle have characteristic patterns during the dynamic and static periods of stretch In order to further analyse the mechanisms underlying these patterns we have studied the sensitivity of the spindle to an interjected brief test stretch The sensitivity of the spindle in terms of the threshold stretch for eliciting a spike or of the amplitude of the receptor potential increased during dynamic stretch and also increased with faster rates of stretch During the dynamic static transition period the sensitivity fell so that at higher levels of extension no response to the test stretch could be elicited The sensitivity during the static phase was lower than during the dynamic phase at the same level of stretch Following the termination of stretch the sensitivity of the spindle underwent a long period of depression which increased with the amplitude and duration of the previous stretch These changes in sensitivity may possibly be related to the mechanical properties of the intrafusal muscle fibres or to the electrical properties of the nerve terminals

Ottoson D and G M Shepherd Receptor potentials and impulse generation in the isolated spindle during controlled extension *Cold Spr Harb Symp quant Biol* 1965 *30* 105—114
Shepherd G M and D Ottoson Response of the isolated muscle spindle to different rates of stretching *Cold Spr Harb Symp quant Biol* 1965 *30* 95—103

* NATO Postdoctoral Fellow of the National Science Foundation
** Special Fellow of the U S Public Health Service

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Mellander S and O Lundgren (Department of Physiology University of Goteborg Sweden) FACILITATION OF TISSUE CLEARANCE BY TRANSCAPILLARY FILTRATION AND ABSORPTION

The effect of net transcapillary fluid movement on the rate of tissue clearance of I^{131} Rb^{86} and Xe^{133} was studied in cat skeletal muscle A technique was used which permitted control directly or indirectly of other factors of importance for exchange, such as blood flow flow velocity capillary flow distribution size of capillary surface area and capillary permeability Net fluid filtration was produced by raising mean hydrostatic capillary pressure and net fluid absorption by increasing plasma osmotic pressure

It was shown that the presence of a net transcapillary fluid movement *per se* (filtration or absorption) increased the clearance rate considerably above the control level when no net fluid movement occurred The clearance rate seemed to increase progressively with increasing rates of net fluid movement at least in the lower range of fluid transfer This relationship was most clearly demonstrated for Rb^{86} during filtration The percentage increase in clearance above control was 2 to 4 times greater for Rb^{86} than for I^{131} during filtration while during absorption the increase was if anything less pronounced for Rb^{86} than for I^{131} Net fluid filtration increased the clearance of Xe^{133} above control but the percentage increase appeared to be less pronounced than for I^{131} A reduction in nutritional blood flow is followed by a decrease in clearance rate It was found that this decrease was much less pronounced where the reduction in flow associated with a net transcapillary fluid filtration

Most vascular reactions in skeletal muscle are known to be accompanied by changes in net transcapillary fluid movement secondary to shifts in the pre to postcapillary resistance ratio In view of the present results this particular vascular response pattern might be of considerable importance in facilitating the blood tissue exchange

Meyerson B A (Department of Physiology Karolinska Institutet Stockholm Sweden) AN ELECTROPHYSIOLOGICAL STUDY OF THE DEVELOPMENT OF INTERHEMISPHERIC FUNCTIONS

The ontogeny of the functional interdependence of the two cerebral hemispheres has been studied during the pre and postnatal periods in sheep In the adult the electrophysiological signs of activity correlated to interhemispheric functions are considered to be mediated both via the cerebral commissures and via the brain stem In the present investigation the development of electrically induced interhemispheric responses and the bilateral synchrony of the spontaneous cortical activity were studied An interhemispheric response could for the first time be recorded at a foetal age of about 65 days (full term 150 days) This response consisted of a positive negative sequence of potentials which were of the same general configuration as those found in the adult animal although the latency and duration were much longer and the stimulus threshold considerably higher Recordings of cortical responses to callosal stimulation as well as callosal responses to cortical stimulation indicated that this foetal interhemispheric response was mediated through the corpus callosum During the earliest phase of development the response could only be obtained from the fronto-parietal areas of the brain at that time lissencephalic Later during maturation the cortical responsiveness to contralateral stimulation was found to proceed in a lateral and posterior direction At about the 85th day this interhemispheric response was followed by a late component sometimes appearing as a separate sequence of potentials with a remarkably long latency The characteristics of this late response indicated that it is mediated via extra-callosal pathways

The development of the bilateral synchrony of the spontaneous cortical activity was studied by means of cross-correlation analysis performed on a digital computer (Meyerson and Moller in preparation) The correlograms so far obtained from the youngest foetuses (60—75 days) in which spontaneous activity could be recorded showed two definite maxima symmetrically around zero-delay These results may imply that already in the earliest phase of EEG-development there is a certain interdependence of cortical activity within the two hemispheres However the absolute values of the correlation coefficients seem to remain rather low during the whole developmental period

Mårtensson A. (Department of Physiology Karolinska Institutet Stockholm Sweden) ON THE POSSIBLE EXISTENCE OF DOUBLY INNERVATED MUSCLE FIBRES IN INTRINSIC LARYNGEAL MUSCLES

Experiments were performed on the cricothyroid and thyroarytenoid muscles of anesthetized dogs with the object of searching for evidence indicating the presence of doubly innervated muscle fibres

On the *cricothyroid* which is supplied by two peripheral nerves three types of experiment were performed *1)* When tetanic stimulation was applied to the nerves together respectively individually the tension developed on combined stimulation showed no deficit as compared to the sum of the tensions produced on individual stimulation Since any muscle fibre supplied by both nerves should develop the same tension on combined as on individual stimulation this finding is evidence against overlap of innervation 2) When a single shock applied to one of the nerves was followed by a similar shock to the other nerve at an interval sufficient for any fibre supplied by both nerves to contract twice and consequently to develop more tension than on simultaneous stimulation the recorded peak tension proved to be the same as on simultaneous stimulation thus providing further evidence against overlap of innervation 3) When subtetanic stimuli of identical frequencies (25/sec) were applied to both nerves the tension developed was the same when corresponding volleys in the two nerves reached the muscle in phase respectively sufficiently out of phase to permit any fibre supplied by both nerves to increase its discharge frequency and its tension development consequently also these experiments failed to provide evidence for polyneuronal innervation

The *thyroarytenoid* is supplied by only one peripheral nerve but by selective blocking of the motor axons experiments of types 2) and 3) could be performed also on this muscle However no conclusive evidence for the existence of doubly innervated fibres was obtained

Makinen K. (Institute of Dentistry University of Turku Finland) HYDROLYTIC ENZYMES OF HUMAN SALIVA AND PLAQUE

Peptidase esterase sulphatase phosphatase and glucosidase activities in the whole human saliva and plaque were studied The materials were fractioned on Sephadex G-100 G 200 and DEAE columns and the enzyme activities were tested using the following substrates 26 different amino acid β naphthylamides for aminopeptidases N-carbo-β naphthoxy DL-phenylalanine for carboxypeptidases denatured haemoglobin collagen native casein lactalbumin or serum albumin for endopeptidases β-naphthyl acetate propionate and valerate for esterases 6-bromo-β-naphthyl sulphate for sulphatases 6-bromo-β naphthyl-α D-glucoside for glucosidases and various naphthylphosphates for phosphatases

Fractionation of the test material revealed a great number of different enzyme activities indicating the existence of a rich enzyme spectrum in the human saliva Experiments performed simultaneosly on plaque demonstrated that the majority of these enzymes arose from the oral bacteria

All the enzymes observed had a molecular weight of about 100000 or more The enzymes also possessed closely similar activities between pH 6 and 8 provided that the enzymes were saturated with the substrate The oral cavity seemed to contain several enzymes of rather low specificity as well as some with strictly specific properties e g a prolinase with a molecular weight of about 90000 The substrates containing aliphatic and hydrophobic side chains were cleft by the largest number of enzymes those bearing strongly acidic or basic residues or aromatic rings were cleft only by a few Among the peptidase substrates those containing N terminal L-alanyl or L-prolyl residues were hydrolyzed most rapidly No metal cation has so far been found to activate any of these peptidases or esterases As to the dental caries and periodontal diseases it is worth noticing that the oral plaque produces both endopeptidases capable of hydrolytizing native and denatured protein molecules and phosphatases capable of hydrolyzing various phosphate esters The endopeptidases found in the dental plaque seemed stable against prolonged incubations at +37° C Accordingly they may have the same stability also in their place of formation in the plaque

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Møller, A R C G Bernhard and W H Miller (Department of Physiology Karolinska Institutet Stockholm Sweden) THE INSECT CORNEAL NIPPLE ARRAY AS AN OPTICAL IMPEDANCE TRANSFORMER RELATION BETWEEN NIPPLE FORM AND EFFICIENCY

The insect corneal nipple array found in electromicroscopic studies (Bernhard and Miller 1962) acts as a broad band impedance transformer to increase light transmission and decrease reflection as shown in microwave model experiments and spectrophotometric investigations (Bernhard *et al* 1963 1965 Miller *et al* 1964 1966) The mechanism of the action of this regular lens surface structure the anlage of which can be seen early in the pupal stage (Gemme 1966) was also demonstrated by numerical calculations performed on a digital computer on the basis of a simplified pattern consisting of geometrical cones (Bernhard *et al* 1965) Calculations have now been made to show how changes in the shape of the nipples influence their efficiency in reducing the surface reflection in various parts of the visible spectrum The results of these studies will be presented and the manner in which the nipple efficiency depends upon the size density and form of the nipples will be discussed

Bernhard C G and W H Miller *Acta physiol scand* 1962 *56* 385—386
Bernhard C G W H Miller and A R Moller *Acta physiol scand* 1963 *58* 381—382
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Miller W H C G Bernhard and A R Moller *J Opt Soc Amer* 1964 *54* 581—582
Miller W H A R Moller and C G Bernhard in *The Functional Organization of the Compound Eye* (Ed C G Bernhard) Wenner Gren Cent Internat Symp Vol 7 1966 In press

Naeraa N E Boye and E Strange Petersen (Department of Physiology University of Aarhus Denmark) THE INFLUENCE OF VENTILATION /PERFUSION DISTURBANCES ON HELIUM AND CARBON DIOXIDE CURVES RECORDED FROM UNEVENLY VENTILATED LUNGS

From a theoretical point of view the combination of a normal individual breath helium curve (heliogram) and an abnormal carbon dioxide curve (capnogram) should not exist However it has now been observed in patients several times In order to test the hypothesis that this combination is created mainly by perfusion disturbances a lung model was constructed Airway resistance compliance and perfusion (inflow of CO) of the two lungs could be varied independently Helio- and capnograms were recorded from the mouth and from the main bronchi while the relations between resistance compliance and perfusion were changed systematically By means of a Stephenson respirator each set of experiments was performed at two respiratory frequencies 15 and 30 per minute

With normal compliance values a threefold decrease of the diameter of one main bronchus did not create a slope in the alveolar part of the heliogram at the low frequency If perfusion was equal in the two lungs the capnogram showed no slope If however perfusion was present on the stenosed side only a marked positive slope appeared in the capnogram On the other hand a negative slope was created with opposite perfusion conditions Thus the combination described clinically could be produced by experiment but only at the low respiratory frequency At the higher frequency both curves became abnormal

With normal conditions present in both lungs normal curves were obtained at both frequencies

Nicolaysen K. (Neurophysiological Laboratory Anatomical Institute University of Oslo Norway) ON THE FUNCTIONAL PROPERTIES OF THE FAST AND SLOW CRANIAL MUSCLES OF THE ATLANTIC HAGFISH

The cranial muscles of the Atlantic hagfish offer certain advantages for the study of the properties of the fast and slow skeletal muscle fibres Contrary to the trunk muscles where the fast and slow muscle fibres are intermingled there are some cranial muscles which consist of fast and slow fibres exclusively

The m longitudinalis linguae consist solely of fast fibres Each such muscle fibre is innervated by a single axon terminating in a localized end plate On indirect stimulation the characteristic endplate potential and propagated action potential are recorded from the muscle fibres The peak time of the contraction is approximately 60 msec and the tetanic fusion frequency is approximately 50 cps

The m spinovelaris and m craniovelaris posterior consist exclusively of slow muscle fibres Each muscle fibre is usually innervated by more than 5 axons As revealed by the spontaneous junction potentials the synapses appear to be distributed along the entire length of the muscle fibres Histological observations indicate that the synaptic sites are approximately 10 μ apart The number of muscle fibres in the m spinovelaris is approximately 5 times the number of axons supplying the muscle

On indirect stimulation the slow fibres produce junction potentials that summate but never reverse the membrane potential The time course of decay of the junction potentials consists of an early rapid and a later slow component A single shock to the nerve produces only a small contraction A second shock within 20 msec produces a much larger contraction The time to peak contraction is about 125 msec The tetanic fusion frequency is appoximately 35 cps but the tension continues to increase with increasing frequency of stimulation up to 150 cps

Nielsen K. H (Department of Biochemistry University of Aarhus Denmark)

INHIBITION OF PHENYLALANINE 4-HYDROXYLASE FROM RAT LIVER

The enzyme system phenylalanine hydroxylase (1 99 1.2) which catalyzes the hydroxylation of L phenylalanine to L-tyrosine as shown by Kaufman (1) (2) consists of at least 2 enzymes and 2 co-factors which react after the following scheme

I L phenylalanine+O +2-amino-4-hydroxy-6-alkyl tetrahydropteridine ⟶
L tyrosine+2 amino-4 hydroxy-6-alkyl-dihydropteridine+H O

II 2 amino-4-hydroxy-6-alkyl-dihydropteridine+NAD(P)H+H⁺ ⟶
2 amino-4-hydroxy-6-alkyl tetrahydropteridine+NAD(P)⁺

Specific inhibition of phenylalanine hydroxylase is desirable for the investigation of L-phenylalanine metabolism under conditions similar to those found in patients suffering from phenylketonuria Several compounds inhibiting this enzyme system are known (3) (4) but as to whether it is process I or II or both which are inhibited by these compounds little is known

The enzyme activity of process I can be determined by measuring the tyrosine formation after 4 minutes reaction time in a solution which shaken in an open tube at 30 C contains phosphate buffer pH=6 8 (0 1 M) ascorbic acid (10 mM) 2 amino-4-hydroxy-6-methyl tetrahydropteridine (0 03 mM to 0.3 mM) glucose dehydrogenase (1 1 1 47) phenylalanine hydroxylase I and L-phenylalanine (2 mM) Since the tetrahydropteridine is both consumed by the hydroxylation reaction and oxidized by atmospheric oxygen *the presence of ascorbic acid in the solution is essential* to keep the concentration of this compound at a constant level (95 % to 98 % of the total hydropteridine) A partial inactivation of the enzyme (phenylalanine hydroxylase I) is avoided when glucose dehydrogenase is present in the solution

The enzyme activity of process II can be determined by measuring the decreasing rate of concentration of NAD(P)H in a solution containing tris buffer pH=6 8 (0 1 M) H O (10 mM) horse raddish peroxidase 2 amino-4-hydroxy-6-methyl-dihydropteridine (0 005 mM to 0 1 mM) phenyl alanine hydroxylase II and NAD(P)H (0 1 mM to 0.2 mM) H O + peroxidase keeps the dihydropterine (added to the solution as tetrahydropteridine) oxidized during the measurement of the enzyme activity and this oxidation system is the only one known at present which is capable of oxidizing the tetrahydropteridine to dihydropteridine without oxidizing NAD(P)H simultaneously when phenylalanine hydroxylase II is present

Inhibition of these 2 enzyme reactions are measured at different concentrations of inhibitors hydropterines and L-phenylalanine

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Kaufman S B Levenberg *J biol Chem* 1959.234 2683
Ross S B O Haljasmaa *Acta pharmacol (Kbh)* In press

Niemi M and M Ikonen (Department of Anatomy University of Turku Finland) AN INVESTIGATION OF THE ENDOCRINOLOGICAL ACTIVITY IN THE HUMAN FOETAL TESTIS

A series of foetal testes covering an age range from the 7th to the 27th week (CR length 2 4—22 8 cm) was studied a few autopsy testes of premature babies were also included The morphological differentiation of the primitive interstitial tissue began at the 8th week (CR 3 cm) and maximal number of fully differentiated Leydig cells was reached at about the 12th week (CR 12—13 cm) An uninterrupted line of fine structural development could be followed from the primitive fibroblasts of the peritubular mesenchyma to the fully mature foetal Leydig cells The latter were much alike their adult counterparts having an extremely prominent smooth endoplasmic reticulum no crystals of Reinke were visible however Simultaneously with the morphological differentiation an appearance of a number of enzymatic histochemical activities could be demonstrated Special attention was paid to the capacity of the foetal Leydig cells to utilize in a tetrazolium reducing system substrates (NADPH glucose-6-phosphate beta hydroxybutyrate) supposed to have importance in steroidogenesis and steroidal substrates proper (3 beta and 16-beta hydroxysteroids)

Fresh testis tissue from 14.2 to 21 4 weeks old foetuses was also incubated with [7α ^{3}H] progesterone and 17α hyroxy[$7\alpha^3$ H]pregnenolone as substrates The steroid conversion products included 17α progesterone dehydroisoandrosterone androstendione androstendiole and testosterone The yield of testosterone was higher when 17α hydroxypregnenolone was used as the substrate but the relative amount of testosterone converted from both substrates did not vary significantly in testes of different age

Nienstedt W and K. Hartiala (Department of Physiology University of Turku Finland) INTESTINAL METABOLISM OF ABSORBED PROGESTERONE

Microgram amounts of 4-C progesterone were placed into isolated intestinal segments of narcotized heparinized dogs The effluent venous plasma was deproteinized with ethanol and fractioned with Florisil chromatography into lipid free steroid steroid sulphate and steroid glucuronide fractions The free steroid fraction contained essentially all of the radioactivity of the original whole blood sample It was chromatographed bidimensionally on a silica gel thin layer plate at +6 C with methyl acetate-ethylene dichloride (30 70 v/v) and hexanol hexane (55 45 v/v) Hydroxysteroids are preferentially retained in the former ketosteroids in the latter solvent system Autoradiography was used in the localization of radioactive compounds

In addition to progesterone about twenty different metabolites were regularly found in the effluent plasma collected within half an hour after the application of the radioactive progesterone Several of them were detectable in the first five minutes The share of unchanged progesterone diminished with time so that the combined radioactivity of the metabolites was greater after about fifteen minutes Some of the metabolites were tentatively identified as various reduction products (especially of the 5α-series) of progesterone Only a small portion of the metabolic effects can be explained on the basis of microbial action

Niinikoski J R Penttinen and E Kulonen (Department of Medical Chemistry University of Turku Finland) EFFECT OF OXYGEN SUPPLY ON THE TENSILE STRENGTH OF HEALING WOUND AND OF GRANULATION TISSUE

Viscose cellulose sponges (*two* pieces 20x10x10 mm each) were implanted subcutaneously in the backs of adult rats The animals were kept for ten days in an atmosphere wich contained 21 % (air) 50 % or 100 % of oxygen The rats were then killed and the tensile strengths (TS) in the healing skin wounds and in the granulomas were determined and calculated as described by Viljanto (Acta Chir Scand Suppl 333 1964) The following results were obtained

21 % 0 TS in wounds 349 6±38 1 (11) in granulomas 93 1±12 3 (8)
50 % O_2 TS in wounds 439 1±25 6 (16) in granulomas 99 7± 8 7 (15)
100 % O_2 TS in wounds 509.5±30 6 (10) in granulomas 128 5± 7 7 (10)

The standard errors of the means and the number of the rats are indicated The variances between the groups were larger than within the groups (P<0 02, almost 0 01 for wounds and P<0 05 for granulomas

Supported by a grant from Sigrid Juselius Foundation

Nilsson G (Department of Pharmacology Karolinska Institutet Stockholm Sweden) DUODENAL INHIBITION OF GASTRIC ACID SECRETION IN RESPONSE TO VAGAL STIMULI

The pH sensitive duodenal mechanism responsible for the inhibition of gastric secretion has been localized mainly to the duodenal bulb The mechanism is highly effective in suppressing gastric secretion stimulated by exogenous gastrin (Andersson Nilsson and Uvnas 1965)

In the present series of experiments the effectiveness of the bulbar inhibitory mechanism was studied on secretory responses to insulin hypoglycaemia and sham feeding The experiments were performed on Pavlov pouch dogs with isolated pouches of the duodenal bulb The duodenal bulb was acidified by perfusion with 0 1 N HCl and buffer solutions of different pH for periods varying between 5 to 60 minutes

Gastric secretion in response to insulin hypoglycaemia was inhibited when the intrabulbar pH was reduced to 1,2—1 4 Responses to lower doses of insulin (0 1—0,2 U/kg) were abolished while responses to larger doses were less influenced

Gastric secretion in response to sham feeding was abolished with an intrabulbar pH of 1,2—1 4 The period of inhibition was dependent upon the time of acidification Reduction of the intrabulbar pH to 1,2—1 4 for 5 minutes produced nearly complete inhibition for about one hour

Previous experiments have shown that gastrin stimulated secretion is inhibited by a bulbar pH of 2,5 or lower However secretory responses to sham feeding are more susceptible to duodenal inhibitory influences since a considerable inhibition was observed at a bulbar pH of 3—3,5

Further experiments are being planned to elucidate the duodenal inhibitory influence on vagally induced gastric secretion Combined with earlier observations (Andersson Nilsson and Uvnas 1965 Andersson and Grossman 1965) the present results strongly indicate that inhibitory influences arising from the duodenal bulb could play an important role in the normal regulation of gastric acid secretion

Andersson S and M I Grossman Profile of pH pressure and potential difference at the gastroduodenal junction in man *Gastroenterology* 1965.364—371

Andersson S G Nilsson and B Uvnas Inhibition of gastric secretion by acid in proximal and distal duodenal pouches *Acta physiol scand*, 1965 65 191—192

Nissen O I (*Institute of Medical Physiology B University of Copenhagen Denmark*) THE FILTRATION FRACTIONS OF PLASMA SUPPLYING THE SUPERFICIAL AND DEEP VENOUS DRAINAGE AREA OF THE CAT KIDNEY

In 23 experiments the plasma concentrations of protein (P) and inulin (I) were measured in blood collected simultaneously from an artery from the subcapsular and from the deep veins of the cat kidney These concentrations allow a calculation of the filtration fractions (FF) of the plasma flows supplying partly the superficial cortex and partly the deep cortex plus medulla If the plasma concentrations of a substance X in arterial blood and renal venous blood from a drainage area are called A_X and V_X respectively the formula for the filtration fraction of the area is

$$FF = \frac{1}{f_T} \cdot \frac{V_I/A_I - V_P/A_P}{V_I/A_I}$$

where f_T $(=1.05)$ is a factor which corrects a concentration of a substance in plasma to the concentration in plasma water

A filtration fraction averaging 0.34 was found for the plasma supplying the superficial cortex The filtration fraction of the plasma supplying the deep cortex and the medulla was lower the ratio filtration fraction in deep area/filtration fraction in superficial area was usually 0.70 to 0.95 and the average (0.85) was significantly lower than 1.00 ($p<0.001$)

The existence in the deep parts of the kidney of various vessels through which the plasma (blood) may by pass the capillary tufts of juxtamedullary glomeruli provides the possibility of accounting for the observed deviation of the ratio from 1.00 without resorting to the assumption of (basically) different filtration fractions for plasma entering glomerular tufts in deep and superficial glomeruli A by pass of a magnitude sufficient to account for the deviation may also account for a medullary plasma flow as reported by others

Norberg K A and B Fredricsson (Department of Histology Karolinska Institutet Stockholm Sweden) CELLULAR DISTRIBUTION OF MONOAMINES IN THE UTERINE AND TUBAL WALLS OF THE RAT

The distribution of adrenergic nerves and cells containing serotonin in the uterine and tubal walls of the rat was studied by means of the histochemical method of Falck and Hillarp Adrenergic innervation of the uterine muscle occurs practically only in the part nearest the tubo-uterine junction where the adrenergic nerves form a dense network possibly with a sphincteric function as earlier described for the rabbit (Brundin 1965) The changes in the distribution of cells containing monoamine and fibres as followed during the different parts of the sexual cycle and these data discussed in relation to earlier isotope studies (Wurtman *et al* 1963)

Brundin T *Acta physiol scand* 1965.66 Suppl 259
Wurtman R J E W Chu and J Axelrod *Nature (Lond)* 1963.198.547—548

Norrsell U (Department of Physiology University of Goteborg Sweden)

CEREBRAL SOMATOSENSORY ABLATIONS AND A TACTILE CONDITIONED REFLEX

Dogs were trained to respond to light tactile stimulation of either hind limb by pressing a plate with the right forelimb Correct responses were reinforced with food The effect of cortical lesions was tested both on the training of naive animals and on animals trained preoperatively to maximum performance The lesions consisted of unilateral aspiration of the hindlimb projection areas in the somatosensory cortex (areas SI and SII) which were determined in each case during the operation by the evoked potential technique No differences between the sides were observed in the training of the animals with unilateral lesions However in the preoperatively trained animals the lesions resulted in a transient impairment mainly of those reflexes that were elicited from the limbs contralateral to the lesions After the animals had recovered from the effects of the initial lesions similar ablations of the other previously spared sides were performed The lesions resulted in impairment mainly of those reflexes that were elicited from the limbs contralateral to the freshly made lesions The effects of the second operation were in all instances more severe than those of the first operation The lesions were in no instance observed to affect the motor performance of the conditioned reflex Nor were the enhanced effects of the second operations dependent of whether the lesions were ipsi or contralateral to the limb used for the conditioned response

XII Scandinavian Congress of Physiology Turku 1966
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Ohlin P and E J Perec* (Institute of Physiology University of Lund Sweden) EFFECTS OF REPEATED TEETH AMPUTATIONS ON THE RAT S SUBMAXILLARY GLAND

Repeated amputations of the incisors in rats cause an enlargement of the submaxillary gland which histologically corresponds to a growth of the acinar cells (PEREC *et al* 1964) The hypertrophy of the glands has been considered to be due to a reflex via the glandular nerves (HOUSSAY *et al* 1962 WELLS and PERONACE 1964) In the present investigation particularly the role of the parasympathetic nerve fibres has been studied in addition the secretory function of enlarged glands was estimated

The increase in the weight of the submaxillary gland after repeated teeth amputations was markedly reduced after parasympathetic denervation either preganglionic surgical denervation by section of the chorda lingual nerve or postganglionic pharmacological denervation by treatment with atropine Sympathetic denervation had a similar effect The weight increase was prevented by combined parasympathetic and sympathetic denervation

The choline acetylase activity (expressed as concentration) did not change in spite of the glandular enlargement When expressed as total activity a 54 % increase in the hypertrophic gland was found thus suggesting an extension of parasympathetic innervation When parasympathetic denervation was combined with teeth amputation the same results were obtained The effect on the parasympathetic neurones is not dependent on an intact innervation The concentration was increased after sympathetic denervation (Nordenfelt 1964) no changes were obtained when combined with incisor amputations

Secretion of saliva was studied after intravenous injections of sialagogue agents or stimulation of the chorda lingual nerve The secretory responses of enlarged glands to parasympathomimetics such as acetylcholine and methacholine did not differ from those of control glands Similarly the maximal secretory capacity to chorda stimulation was found to be unchanged in spite of the enlargement of the acinar cells On the other hand the secretory responses of enlarged glands to sympathomimetic agents *e g* adrenaline were markedly increased

Perec C A H Houssay A Peronace and J Fiedotin *J dent Res* 1965 44 683
Houssay A B A Peronace C Perec and O Rubinstein *Acta physiol lat amer* 1962.12 153
Wells H and A. Peronace *Amer J Physiol* 1964.207.313
Nordenfelt I *Acta Univ Lund* 1964 II No 10

*) Fellow of the Consejo Nacional de Investigaciones Cientificas y Tecnicas Buenos Aires — Argentina

Oja S S T Hamalainen and S Pajunen (Institute of Physiology University of Helsinki Finland) A STATISTICAL MULTIVARIATE ANALYSIS OF NUCLEOTIDES IN THE DEVELOPING RAT BRAIN

The free nucleotides of the developing rat brain were separated by column chromatography There was a decrease in the concentration of triphosphates and creatine phosphate and an increase in the level of diphosphates and especially monophosphates with increasing age (Oja 1966)

To investigate the sources of variance in the nucleotide pattern the results were subjected to a principal component analysis (*Elliot 803 computer*) The calculations were based on the correlation matrix of the 14 variables measured The age component amounted to 63 % of the total variance The following four components accounted for 27 % and the remaining 10 % was distributed among the last nine components Age was partialled out and the final analysis performed by means of the matrix of partial correlation coefficients The extracted components were rotated orthogonally (Varimax criterion) A rotated solution with four components was biologically meaningful in the best manner The first component was associated with a high level of adenosine and guanosine triphosphates the second with the stage of phosphorylation of uracil nucleotides the third had high positive saturations in creatine phosphate and adenosine diphosphate and the fourth a high negative saturation in nicotinamide adenine dinucleotide A factor analysis of the nucleotides was also performed It gave similar results The present analyses may be compared with another one made on similar data with altogether 45 biochemical variables (Oja 1966)

Oja S S Postnatal changes in the concentration of nucleic acids nucleotides and amino acids in the rat brain *Ann Acad Sci fenn* A 5 1966 125 In press

Olson L. (Department of Histology Karolinska Institutet Stockholm Sweden) EXCESSIVE GROWTH OF ADRENERGIC NERVES IN DUCED BY A SPECIFIC NERVE GROWTH PROMOTING PROTEIN

Growth and differentiation of adrenergic neurons are known to be regulated by a specific nerve growth factor (NGF) occurring in high concentrations in *e g* certain tumors snake venom and submaxillary glands of the mouse (Levi—Montalcini 1964) The effect of NGF has been studied in mice by means of a highly specific fluorescence technique for histochemical demonstration of certain monoamines Newborn albino mice were injected subcutaneously with 0 05 ml/g b w of a highly purified NGF*) 1 to 2 times a day for 5 to 12 days The mice were killed 1 to 7 days after treatment and pieces of iris salivary glands and sympathetic ganglia were taken out and treated for fluorescence microscopy

A striking effect was observed in the iris The number of adrenergic terminals in each strand of the ground plexus was increased from nor mally one single up to 2 to 4 Also the number of non varicose axons had increased considerably In the sublingual gland which normally practic ally lacks parenchymal adrenergic innervation a slight increase in the number of terminals seemed to have occurred The number and size of the ganglion cells seemed to have increased The significance of these results in respect to nerve regeneration dynamics will be discussed

Levi Montalcini R *Ann N Y Acad Sci* 1964.*118* 149—170

*) The author is much indebted to Prof R Levi—Montalcini for generous supplies of NGF

XII Scandinavian Congress of Physiology Turku 1966
Acta physiol scand 1966 68 Suppl 277

Paalzow L. (Apotekens Centrallaboratorium Solna Sweden) QUANTITATIVE ESTIMATION OF ANTICONVULSANT DRUGS IN MICE

A new electroshock procedure is described This is based on maximal electric stimulation of the brain of mice for 0 4 sec by temporal electrodes using square wave current having a pulse interval of 1.25 10^{-3} sec and a pulse width of 0 6 10^{-3} sec

Because of the carefully standardized electric shock only 8—12 volts are needed to elicit convulsions The tonic extensor phase of the hindlegs is taken as a sign of convulsant effect

The procedure differs from most of the methods previously described by taking the convulsion threshold of each animal into account Thus it is possible to expose each mouse repeatedly to a sublethal electric potential which gives the possibility to follow the effects from time to time in each animal

The log dose response lines of some well known antiepileptic drugs will be discussed as well as the possibility of evaluating new anticonvulsant substances

Paasilainen T ■ A and L. Hirvonen (Cardiorespiratory Research Unit Department of Physiology University of Turku Finland) RESPONSE OF PROPRANOLOL PRETREATED ISOLATED ATRIUM OF A HYPOTHYROID AND THYROXINIZED RAT HEART TO ADRENALINE AND NORADRENALINE

Both adrenaline and noradrenaline have a positive chronotropic and inotropic effect on the heart muscle in an isolated state not only in normal condition but also in hyperthyroid and hypothyroid preparations Whether these responses are changed by a β receptor blocking agent (propranolol = Inderal I C I) was studied on an isolated atrial preparation of the rat (20 normal 40 l thyroxine or tri iodo-l thyronine treated and 15 radiothyroidectomized animals) The spontaneous beat rate and isometric contraction force of the preparation were recorded at 30 C with an apparatus consisting of a lever resting on a rubber membrane of a water filled chamber coupled to an Elema pressure transducer The Inderal dose in the Locke s solution bathing the preparation was 1×10^{-3} mg/ml After 15 minutes adrenaline or noradrenaline was added to make a concentration of 1×10^{-5} 1×10^{-4} 1×10^{-3} and $1\times10^{-}$ mg/ml In addition to this Inderal was injected intravenously into 24 animals before the preparation was made

Inderal decreased the beat rate in all preparations The contraction force did not change significantly Adrenaline and noradrenaline did not increase the beat rate of euthyroid and hyperthyroid Inderal treated preparations The beat rate of hypothyroid preparations increased The response was equal to both substances The contraction force after smaller adrenaline and noradrenaline doses increased less in the Inderal series than in the controls With the highest dose an equal response was obtained in both series Adrenaline and noradrenaline gave similar results in the euthyroid and hyperthyroid series In the hypothyroid series adrenaline increased the contraction force more than noradrenaline There were no differences between the results of experiments where Inderal was administered *in vitro* and those where it was given *in vivo*

XII Scandinavian Congress of Physiology Turku 1966
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Paavilainen T O A, L. Hirvonen and T Peltonen (Cardiorespiratory Research Unit Department of Physiology University of Turku Finland)
SUGAR CHOLESTEROLE AND CREATININE CONCENTRATIONS IN THE FOETAL BRONCHIAL FLUID

The concentration of different substances in the foetal bronchial fluid is like in the amniotic fluid a result of dynamic equilibrium The bronchial fluid is an intermediate zone in the transfer between the amniotic fluid and the foetal respiratory organs The concentrations of representatives of carbohydrates (sugar) lipids (cholesterol) and waste products of protein metabolism (creatinine) were determined in the amniotic fluid the foetal bronchial fluid and the blood of the mother animal and foetus Sven sheep with 15 foetuses and 11 guinea pigs with 42 foetuses served as test animals Cesarean section was performed on the sheep under local anesthesia The guinea pigs were slightly anesthetized be Nembutal All the sheep foetuses were near the termination of pregnancy A group of guinea pig foetuses was delivered near term (weight 90 to 115 g) and another group in a less mature state (weight 50 to 70 g) In the sheep the highest sugar concentration was observed in the amniotic fluid and the lowest in the bronchial fluid In the guinea pigs the maternal serum had the highest and the bronchial fluid the lowest sugar content The highest cholesterol concentration in the sheep was that of the maternal serum and in the guinea pigs that of the foetal serum The lowest cholesterol concentration was in the bronchial fluid in both species In the lambs there was in the amniotic fluid ten times as much cholesterol as in the bronchial fluid In the guinea pigs the cholesterol content of the amniotic fluid was as low as that of the bronchial fluid In the guinea pig series the results did not vary with foetal size The creatinine concentration was highest in the amniotic fluid of all animal groups In all the fluids of the guinea pigs with smaller foetuses there was more creatinine than in those of animals with larger foetuses The relative creatinine concentration of the bronchial fluid was as follows Lamb amniotic fluid>bronchial fluid=foetal serum Guinea pig with larger foetuses amniotic fluid=foetal serum>bronchial fluid Guinea pig with smaller foetuses amniotic fluid>bronchial fluid>foetal serum

Pekkarinen, A., K. Manninen and B. Thomasson (Department of Pharmacology, University of Turku, Finland): EFFECT OF CHLORPROTHIXENE, CHLORPROMAZINE AND AMITRIPTYLIN ON THE CONTENT OF ADRENALINE AND NORADRENALINE IN THE ADRENAL VEIN PLASMA OF DOGS DURING IRREVERSIBLE HAEMORRHAGIC SHOCK.

16 dogs, pretreated with tubocurarine, were anesthetized with 60—100 mg/kg of chloralose. Haemorrhagic shock, induced by bleeding from the femoral artery into a reservoir placed at a height equivalent to a pressure of 50 mmHg, caused a 3—35 fold increase of the content of adrenaline in the plasma of the left adrenal vein with the parallel increase of adrenaline secretion (μg/kg/min.) from the "basal" content (40—510) μg/l) before the shock to 1050—7400 μg/l during the shock. The effect of haemorrhage was immediate. The peak of the increase of adrenaline content and secretion was reached within 6—90 min. At the same time a variable increase follows in the content of noradrenaline. The basal content was also influenced by the tubocurarine pretreatment, chloralose anesthesia and the operative trauma caused by the cannulations. 1—2 doses of chlorprothixene (5—10 mg/kg/ i.v.) usually reduced the elevated content of adrenaline and noradrenaline during the haemorrhagic shock or their secretions to the level of the "basal" secretion for 1.5—3 hrs. Further decrease of the arterial blood pressure in the agonal stage of shock in dogs caused a remarkable increase in the adrenaline content of the plasma in the adrenal vein to 1400—14000 μg/l in spite of further administration of chlorprothixene. If the blood flow was then very slow, the secretion of adrenaline was only small or slightly elevated. Our results indicate that chlorprothixene can reduce the adrenaline and noradrenaline content and their secretion at a certain level of severe experimental haemorrhagic shock in dogs, while its effects are lacking during the late agonal stage of the experimental shock with very low blood pressure or are only partial. Either amitriptyline or chlorpromazine can decrease or prevent the increase of adrenomedullary secretion in hemorrhagic shock. Hemaccel® also can reduce the content of adrenaline in adrenal vein of hemorrhagic shock by its hemodynamic action. Only small doses of neuroleptics should be used. High doses of neuroleptics decrease the blood pressure.

Pekkarinen A and U K Rinne (Department of Pharmacology and Neurologic Clinic University of Turku Finland) COMPARISON OF DIFFERENT LONG ACTING CORTICOTROPHIN PREPARATIONS IN THE TWO-DAYS ACTH TEST IN MAN

The adrenocortical responses and their dose relationships to poly phloretin phosphate (I Reacthin®) carboxymethyl cellulose (II Acton prolongatum®) zinc hydroxide (III Cortrophine Z®) and gelatine (IV Depo-ACTH®) corticotrophins (ACTH) were studied in the two-day intra muscular ACTH test (doses 20—120 IU twice daily)

According to the size of the maximum adrenocortical responses to great doses of ACTH (60 or 120 IU) on the second ACTH-day the order of the intensity of the responses was the following I II III and IV

(1) On the second ACTH-day 40 IU of I caused the maximal mean response of excretion per 24 hrs above the mean basal excretion +49.5 mg Porter—Silber 17-OHCS +60.2 mg ketogenic 17 OHCS and +12 1 mg 17 KS 60 IU caused nearly similar responses

(2) The mean response to 60 IU of II (+49 1 mg +67 1 mg and +11 9 mg respectively) was nearly of the same size as that to 40 and 60 IU of I

(3) The mean response with 120 IU of the new type of III (+52 2 mg +51 7 mg and +15 0 mg respectively) corresponded to that with 40 or 60 IU of I and that with 60 IU of II showing a higher mean response only for 17 KS It was not significantly ($p>0\,05$) greater than that with 40 IU of the old type of III (+43 7 mg +44.5 mg and +10.5 mg respectively) The response with III continues clearly longer than that with I II and IV on the day following the 2 ACTH-days indicating that III gives a longer adrenocortical response than I II or IV

(4) The mean response with 120 IU of IV (+37 7 mg +39.5 mg and +10.2 mg respectively) was smaller than with 120 IU of III Only the mean excretion of Porter—Silber 17-OHCS with 120 IU of IV was on the second ACTH-day significantly smaller than with 120 IU of III ($P<0\,05$) The response with 60 IU of IV was only about a half of the corresponding responses of 40 or 60 IU of I and 60 IU of II

The adrenocortical responses caused by 20 IU with III and II were somewhat higher than with I and IV on the second ACTH-day However the excretion of P S 17-OHCS was significantly higher only between III>I ($p<0\,01$) III>IV ($p<0\,01$) and that of 17 KS between III>I ($p<0\,01$) and II>I ($p<0\,01$)

Our results show that there are great differences in the clinical activities of corticotrophins with various long acting substances A unification of the long acting ACTH preparations are needed The present Pharmacopoea methods estimate only the activities of water soluble or gelatine ACTH preparations The excretion of urinary steroids in the two-days ACTH test do not always increase in clear linear proportion of logarithmic scale (e g difference between 20 and 40 I U of III and between 40 and 60 I U of I) Metabolic and absorption factors cause a certain variability in the size of the adrenocortical reserves when they are compared at different dose levels of ACTH preparations which is important clinically to understand in the evaluation of adrenocortical function and in the treatment of patients

XII Scandinavian Congress of Physiology, Turku 1968
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Penttilä, O. and L. Klinge (Department of Pharmacology, University of Helsinki, Finland) ACTION OF ACETYLCHOLINE, BIOGENIC AMINES AND POLYPEPTIDES ON VASCULAR TONUS AND OUTFLOW RATE OF PERFUSED BOVINE ERECTILE TISSUE.

The perfusion tests were carried out by using bovine erectile tissue. Modified Locke's solution was perfused into the dorsal artery of the penis by means of a Dale-Schuster pump. Changes in the infusion pressure after the addition of drugs and the outflow rate from one of the dorsal veins of the penis were recorded.

In most cases no response to acetylcholine (0.005—1.0 mg) was obtained. If the perfusion pressure initially was high, acetylcholine reduced the vascular tonus concomitantly with an increased outflow. The action of acetylcholine was inhibited by atropine (4.0—6.0 mg).

Noradrenaline and adrenaline (2—20 μg) caused a clear increase of the vascular tonus and decrease of the outflow. An approximate ten- to fiftyfold dose of dopamine was required to produce the same responses. Phentolamine (1 mg) inhibited these actions of catecholamines. After phentolamine adrenaline reduced the vascular tonus. Isoprenaline (10—20 μg) too reduced the vascular tonus and this response was inhibited by DCI (1 mg). Thus both α and β receptors exist in erectile tissue. Tyramine (0.1—0.5 mg) effected a slight rise in the vascular tonus.

5-hydroxytryptamine (2—20 μg) and histamine (20—100 μg) caused an increase in the vascular tonus. Both agents caused a reduction of the perfusion flow although the decreasing effect of histamine was more potent. Methysergid (250 μg) and mepyramine (1 mg) inhibited the effects.

Bradykinin, kallidin, eledoisin, angiotensin, vasopressin and oxytocin caused a moderate increase in the vascular tonus and had a potent outflow rate decreasing effect, especially the plasma kinins.

Petersen, O H and J H Poulsen (Institute of Medical Physiology A University of Copenhagen Denmark) CONCENTRATIONS OF SODIUM AND POTASSIUM IN CAT SUBMANDIBULAR SALIVA AFTER ELECTRICAL STIMULATION

The concentrations of ions in the saliva vary with the secretory rate Slightly different results concerning sodium concentration in relation to flow rate have been found by different investigators and in the work of Thaysen Thorn and Schwartz (1954) the spreading in the results for sodium seems to be greater than that for potassium

Burgen (1956) described transient high potassium concentrations (max 40 meq/l) in the first samples of dog submandibular saliva compared with those obtained after the gland had been secreting for some time (steady state)

In the present work secretion of saliva was elicited by chorda stimulation and saliva was obtained from the cannulated submandibular duct The concentrations of sodium and potassium were determined by flame photometry using lithium as an internal standard

Steady state for potassium concentrations was established after a few minutes In typical experiments the sodium concentrations in the first samples (stimulation frequency 10—15 c/sec) were significantly higher than those obtained later at the same secretory rate Maximal transient sodium concentrations were about the concentration in plasma Not until after approximately half an hour of secretion were steady state values of sodium obtained The relation between flow rate and concentration of sodium found in steady state could be reproduced in the same gland after a period of a few hours

Steady state concentrations of sodium generally increased proportionally to the flow rate except at flow rates below 50—100 μl/min/g gland where sodium concentrations were greater than expected on the basis of this relation

Steady state concentrations of potassium were independent of flow rate when this was higher than 100 μl/min/g gland Below this flow rate potassium concentration rose first slightly and then steeply when flow rate decreased and reached intracellular levels (more than 100 meq/l) at flow rates of about 2 μl/min/g gland

Thaysen J H N A Thorn and I L Schwartz *Amer J Physiol 1954 178* 155
Burgen A S V *J Physiol (Lond) 1956 132 20*

XII Scandinavian Congress of Physiology Turku 1966
Acta physiol. scand. 1966.68 Suppl. 277

Piha R. S., R. K. Airas and L. I. Äärl (Department of Biochemistry University of Turku Finland) ACTIVITY OF AMINO ACID sRNA LIGASES IN DEVELOPING MOUSE BRAIN

Different distribution of amino acid activating enzymes in various brain areas was shown by Takahashi and Abe (1963). Previously we found that the rate of incorporation of ^{14}C amino acids into cerebral proteins varied in different brain areas (Piha *et al.* 1963) and that the incorporation rate was more rapid in newborn than in adult (Piha and Uusitalo 1964). To study whether there is any parallelism in the rate of incorporation and in the activity of amino acid activating enzymes the activities of amino acid sRNA ligases were determined during the development of the brain in the mouse from newborn to adult.

The brains were homogenized in the 0.05 M Tris-HCl 0.05 M KCl buffer. After centrifugation (20 000 *g*) the supernatant was passed through Sephadex G-25. This fraction was used for assay of the enzyme activity by ATP PP^{32} exchange method. ATP PP^{32} an amino acid and the enzyme fraction were added to the incubation mixture. After incubation at 37° for 30 min the reaction was stopped with sodium laurylsulphate in 1%. The precipitate was removed and aliquots of the supernatant were applied on Whatman 1 paper for determination of the total radioactivity and for separation of ATP by paper chromatography. The chromatograms were developed ATP spots were marked removed and their radioactivities were measured.

In the adult mouse most of the amino acid sRNA ligases were found in the soluble fraction (105,000 *g*). Microsomes contained the main activity of phenylalanine and some activity of leucine lysine methionine and threonine sRNA ligases.

The activity level of 15 different amino acid sRNA ligases varied in mice of different age to a large extent. The leucine sRNA ligase activity was highest. No activity was found for arginine and glutamic acid sRNA ligases.

In developing brain during the second postnatal week there was a slight but significant decrease in the activity of aspartic acid lysine methionine and threonine sRNA ligases. Decrease in the activity of other enzymes studied was not significant. The results suggest that the activity of amino acid sRNA ligases is not a limiting factor in the rate of protein biosynthesis in the brain.

Piha, R. S. R. M. Bergström L. Bergström A. J. Uusitalo and S. S. Oja, Studies in the metabolism of brain proteins. I. The metabolic turnover rate of brain proteins in the normal rat. *Ann. Med. exp. Fenn.* 1963 41 485—497

Piha R. S. and A. J. Uusitalo Incorporation of ^{14}C-Labelled amino acids into proteins of organs tissues and body fluids in foetal and newborn rats. *Ann. Med. intern. Fenn.* 1964.53.163—178

Takahashi Y. and S. Abe Distribution of amino acid activating enzymes in rabbit s brain. *Experientia (Basel)* 1963.19 156—159

Piha R S and J R Arve (Department of Biochemistry University of Turku Finland) ISOLATION AND CHARACTERIZATION OF MITOCHONDRIAL BASIC PROTEINS FROM BRAIN AND LIVER

The presence of DNA in the mammalian mitochondria has been demonstrated by some workers (Nass *et al* 1965) It is known that in nuclei the histones are associated with DNA and the contents of the two components are approximately the same Therefore an attempt was made to isolate and characterize basic proteins from rat brain and liver mitochondria and compare their concentration with that of DNA

For each experiment three adult rats were used The brains and the livers were homogenized in 0.32 M sucrose solution (pH 6.7) The crude mitochondrial fraction obtained as described earlier (Piha *et al* 1966) was subfractionated in a discontinuous gradient of sucrose with the following steps 1.4 1.2 1.0 and 0.8 M The crude mitochondrial fraction was layered on the top of the density gradient and then centrifuged for 2.5 hours at 38 000 g Four heterogeneous layers and one submitochondrial pellet were obtained Basic proteins were isolated from the mitochondrial pellets by the method used for the preparation of histones from the nuclear pellets (Piha *et al* 1966) DNA was isolated from the mitochondrial residue left after the basic protein extraction according to Schneider and measured by the diphenylamine method

It was found that the quantity of the mitochondrial DNA in the liver was 14.4 μg/g wet weight which agrees with the value of 13.9 μg derived from the data reported by Nass *et al* (1965) The corresponding value for the brain mitochondrial DNA was 10.9 μg The relative amount of basic proteins in mitochondria was several times that of DNA Disc electrophoresis of proteins was carried out on polyacrylamide gels (pH 4.5) The patterns of the basic proteins isolated from mitochondria and nuclei appeared to be quite different which may be an indication of a different type of basic proteins This seems to be supported by the different amino acid composition of the mitochondrial (Rzeczycki *et al* 1963) and nuclear basic proteins

Nass S Margit M K Nass and Ulla Hennix Deoxyribonucleic acid in isolated rat liver mitochondria *Biochim biophys Acta (Amst)* 1965 95 426—435

Piha R S M Cuenod and H Waelsch Metabolism of histones of brain and liver *J biol Chem* 1966 241 2397—2404

Rzeczycki W Alicja Grudzinska M Hillar and Ewa Wszelaki Lass Isolation and amino acid composition of the cathodic protein from hog kidney mitochondria *Acta biochim pol* 1963 9 49—53

Piha R. S L. A Marjanen and H A Jokela (Department of Biochemistry University of Turku Finland) CHROMATOGRAPHIC AND ELECTROPHORETIC ANALYSIS OF SALINE SOLUBLE PROTEINS OF LIVER NUCLEI

Recently it was reported that there is a clear-cut difference in the turnover rates between nuclear basic proteins (histones) and other nuclear proteins of brain and liver (Piha *et al* 1966) The saline soluble and HCl insoluble nuclear proteins have a very rapid turnover Several breaks in their decay curves on semilogarithmic paper are an indication of a heterogeneous mixture of a large number of proteins Therefore it appeared of interest to attempt fractionation of the nuclear proteins with a rapid turnover The present communication deals with the chromatography and electrophoresis of the saline soluble nuclear proteins

The nuclei were isolated from rat and calf liver (Sporn *et al* 1962) and extracted with 0 14 M NaCl (pH 7 0) The extract the saline soluble nuclear fraction was fractionated into three parts The ribosomal fraction was removed from the saline soluble fraction by centrifugation at 105 000 *g* for 120 min During dialysis of the supernatant the globular fraction was precipitated The remaining supernatant was referred to as soluble fraction The three fractions were processed further by Sephadex gel filtration

The results obtained from the ribosomal free supernatant suggest that the globular and soluble fractions may be separated on the Sephadex G-100 column The soluble fraction which consisted obviously of a low molecular weight material is preliminarily fractionated on Sephadex G 75 into two components The globular fraction was separated on Sephadex G-200 and two major peaks were obtained The ribosomal fraction gave rise to two peaks when chromatographed on Sephadex G 100

Disc electrophoresis of proteins was carried out on polyacrylamide gels (Davis 1964) The electrophoretic patterns of the saline soluble nuclear fraction showed at least six bands The mobility of this fraction in acidic circumstances and the electrophoretic analysis of the different subfractions are in progress

Davis B J Disc electrophoresis *Ann N Y Acad Sci* 1964.*121* 404—427

Piha R S M Cuenod and H Waelsch Metabolism of histones of brain and liver *J biol Chem* 1966.*241*.2397—2404

Sporn M B Th Wanko and W Dingman The isolation of cell nuclei from rat brain *J Cell Biol* 1962.*15* 109—120

Piha R S and A Rajamaki (Department of Biochemistry University of Turku Finland) DNA AND HISTONE METABOLISM IN BRAIN AND LIVER *

In the cell nuclei histones are associated with DNA It is generally agreed that DNA is replaced only during cell division In the adult brain cell division is a rare occurrence while in the adult liver a small percentage of the cells is engaged in cell duplication (Post *et al* 1963) It is of interest to compare the metabolic rates of the two components in brain and liver

DNA was labelled with ^{3}H thymidine and histones with ^{14}C-lysine In one group of experiments the isotopic precursors were administered to adult mice Since in the adult due to a smaller number of cell divisions the presence of the labelled components will not be as apparent as in animals labelled *in embryo* in some experiments the isotopic precursors were injected to pregnant mice at a period when a large part of the cell division was still to come

The turnover of DNA and histones labelled in adult and embryo was followed over a period of 9 months Nuclei were isolated and the histones were prepared as described (Piha *et al* 1966) DNA was isolated from the nuclear pellet and from the nuclear residue left after the histone extraction (Piha and Rajamaki 1966) Radioactivity of histones was determined in a gas-flow counter and that of DNA in a Tri-Carp scintillation counter

It was shown that the rates of turnover of DNA and histones labelled in adult and embryo are very similar In the interval between 69 and 150 days the biological half lives of 58—64 days and 58—61 days were calculated for liver DNA and histones respectively This is equivalent to a replacement rate of about 1.2 % per day In the period between 195 and 257 days the half lives correspond to replacement rates of 0.56—0.76 % and 0.62—0.75 % per day for liver DNA and histones respectively Evidence of our previous conclusion that the deoxyribonucleohistone complex is metabolically stable in the nuclei of non-dividing cells and that it is replaced only during cell division is apparent

Piha R S M Cuénod and H Waelsch Metabolism of histones of brain and liver *J Biol Chem* 1966.241.2397—2404
Piha R S and A Rajamaki The rate of turnover of mouse liver DNA compared to that of histones *Suomen Kemistilehti* 1966.B 39 109—111
Post J C Y Huang and J Hoffman The replication time and pattern of the liver cell in the growing rat *J Cell Biol* 1963.18 1—12

*) Part of this study was carried out at Columbia University New York N Y

Pikkarainen P M Koskinen and N Raiha (Research Laboratories of the State Alcohol Monopoly (Alko) Helsinki Finland) DEVELOPMENTAL CHANGES OF ALCOHOL DEHYDROGENASE ACTIVITY IN RAT AND GUINEA PIG LIVER

The activity of alcohol dehydrogenase has been studied in livers of rats and guinea pigs during development The *in vitro* enzyme activity of liver homogenates has been correlated to the ethanol oxidizing capacity of liver slices

In the liver of rat foetuses alcohol dehydrogenase activity is first detected on about the 18th day of gestation At birth the activity is about 25 % of that of the adult and reaches the adult level approx 18 days after birth In the guinea pig liver activity appears about 15 days before term increasing steadily to 20 % of the adult value at birth and then rises rapidly to reach the adult level 7 to 8 days postnatally Ethanol oxidizing capacity of liver slices correlates well with the increase of the *in vitro* enzyme activity in the rat

Administration of ethanol to the pregnant mother before delivery or to the newborn animals after delivery has no effect on the developmental changes of the enzyme activity thus suggesting a lack of substrate induction in this system

The mechanism involved in the developmental increase of alcohol dehydrogenase activity will be discussed in the light of experiments using inhibitors of protein synthesis and steroid hormones

Poulsen J H and O H Petersen (Institute of Medical Physiology A University of Copenhagen Denmark) RESTING AND SECRETORY TRANSMEMBRANE POTENTIALS IN THE SUBMANDIBULAR GLAND OF THE CAT

Lundberg (1958) recorded 3 different kinds of microelectrode responses presumably originating in three different cell types in the submandibular gland of the cat Two of Lundbergs three potential types have been recognized and studied in the present work The secretory potentials were elicited by electrical stimulation of the chorda tympani and close intra arterial injection of acetylcholine (Ach.)

Type I cells (the cells of the acini) have a mean resting potential of −21 mV and hyperpolarize with about 20 mV after stimulation Type III cells have a mean resting potential of about −75 mV and depolarize with about 50 mV after stimulation These results are in accordance with the findings of Lundberg

The effect of varying the stimulation frequency on the size and shape of the secretory potential has been investigated It was found that in type I potentials the degree of hyperpolarization as well as the rate of hyperpolarization increased when the frequency was increased from 2 to 25 c/sec

Graham and Stavraky (1953) showed that injection of large doses of Ach (1000 μg) was followed by a smaller secretion of saliva than injection of more moderate doses (10 μg) Two explanations of this phenomenon seem possible The diminished secretion might be due to an inhibition of the transport processes at the outer acinus cell membrane when Ach in excess of the maximally stimulating concentration is present This would then lead to a diminished secretory potential An alternative possible cause could be that the only reason for the decreased secretion is the diminished blood flow This latter possibility seems likely since we have observed that after intra arterial injection of 10 and 1000 μg of Ach, respectively no difference could be found between the secretory potentials recorded.

Lundberg A *Physiol Rev* 1958 38 21
Graham A R and G W Stavraky *Rev Canad Biol* 1953 *11* 446

XII Scandinavian Congress of Physiology Turku 1966
Acta physiol scand 1966.68 Suppl 277

Pulkkinen M and K Willman (Department of Physiology and Women s Clinic University of Turku Finland) LACTATE DEHYDROGENASES AND ALKALINE PHOSPHATASES IN HUMAN SERUM IN VARIOUS HORMONAL STATES

The levels of lactate dehydrogenases and alkaline phosphatases and their isoenzymes have been studied in the sexes in pregnant women in foetuses and in women under the influence of oestrogens and progestogens (contraceptive pills) to clarify the effect of various hormonal states on these enzymes

The total lactate dehydrogenase activity was determined by following the initial rate of reaction spectrophotometrically For the determination of isoenzymes of lactate dehydrogenase sera were subjected to disc electrophoresis on acrylamide gels The gels were stained by the nitro blue tetrazolium phenazine methosulphate method and the colour intensities of the bonds were measured with a microdensitometer connected to a recorder The activities of the isoenzyme fractions were estimated by weighing the paper between the peaks and the base line

The serum alkaline phosphatase levels were estimated by determining the phosphate liberated from β glycerophosphate by the enzyme under conditions where the activity of the alkaline phosphatase of the human placenta is a maximum

The lactate dehydrogenase level was twice as high in the foetal circulation (umbilical cord) than in the blood of the mother The ratios of the levels of four isoenzymes were the same in both the foetal and maternal sera Pregnancy alone did not alter the total lactate dehydrogenase activity appreciably but the relative ratios of isoenzymes 2—4 changed slightly

The alkaline phosphatase activity was found to be statistically significantly higher in men than in women but was even lower in women who used oral contraceptives The mean activity in the last trimester of pregnancy was four times the mean activity in non pregnant women The alkaline phosphatase level was slightly lower in the umbilical serum than in the maternal serum

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Putkonen P T S (Department of Physiology University of Helsinki Finland) FEAR LIKE BEHAVIOUR ELICITED FROM DORSOMEDIAL THALAMUS AND VENTROMEDIAL FOREBRAIN OF THE CHICKEN

The forebrain and the diencephalon were stimulated in unrestrained White Leghorns to explore various behavioural and motor functions of the avian CNS The methods are described in a previous report (Putkonen 1966) Because of the interest to midline thalamic structures in the emotional behaviour of mammals a special kind of fear response obtained mainly from the avian dorsomedial thalamus is described in this report The thresholds for this response ranged from 0 05—0 35 ma

Typically when the stimulus intensity was gradually raised the birds first looked around and crouched or sneaked as if hiding into a corner of the cage At higher intensities they often made low dashes from corner to corner Restlessness and attempts to escape from the cage were usual after effects Vocalizations often occurred during and after the stimulation

Most of these responses were obtained from electrodes within or near the n dorsomedialis anterior but some similar reactions were elicited from preoptic and septal regions At some septal points the fear response was intermingled with threat behaviour or attacking (Putkonen 1966)

An escape pattern Luftfeind Flucht (von Holst and von Saint Paul 1960) resembling the one described above has been elicited from undefined sites in the chicken brainstem

In cats similar fear like crouching has been elicited from the thalamus mostly from the n medialis dorsalis (Roberts 1962) which nucleus has usually not been regarded anatomically homologous with the avian n dorsomedialis anterior

Holst E von and U von Saint Paul Vom Wirkungsgefuge der Triebe *Naturwissenschaften* 1960 47 409—422

Putkonen P T S Attack elicited by forebrain and hypothalamic stimulation in the chicken *Experientia (Basel)* 1966 In press

Roberts W W Fear like behaviour elicited from dorsomedial thalamus of cat *J comp physiol Psychol* 1962 55 191—197

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Rasmussen, B and K. Klausen (Gymnastikteoretisk Laboratorium Copenhagen Denmark) THE EFFECT OF MECHANICAL RHYTHMICAL PRESSURE CHANGES ON THE RESTING VENTILATION IN MAN

In an attempt to find the somatic afferent neurogenic stimulus for exercise hyperpnea Kao *et al* (1963) exposed the thigh muscles in intact anesthetized dogs and crossed circulation dogs to rhythmical on-off squeezing and found that the ventilation at rest was almost doubled

In our study the ventilation (VE BTPS) oxygen consumption (VO) and the respiratory exchange ratio (R) were investigated in man before during and after a period of rhythmical massage induced A By means of blood pressure cuffs placed on the thighs and B By means of manual massage (Kao *et al* 1963) in our experiments induced on the calf muscles In the experiments with the cuffs the PACO was artificially increased and kept at a constant value throughout the experiment

So far the results from 12 experiments on 3 subjects have not shown a similar effect as seen in the dog experiments The results will be discussed in view of other investigations dealing with passive exercise and the origin of the afferent neurogenic stimulus responsible for exercise hyperpnea

Kao F F C C Michel S S Mei and W K Li Somatic afferent influence on respiration *Ann. N Y Acad Sci* 1963 *109* 696—710

Rasmussen S (Department of Pharmacology Danmarks Farmaceutiske Højskole Copenhagen Denmark) ABSORPTION OF QUININE FROM THE GASTROINTESTINAL TRACT

Tablets containing quinine hydrochloride and coated with cellulose acetas phthalas in layers of varied thickness (0.0336 to 0.242 mg/mm²) were administered to humans and the absorption of quinine was followed by determinations of plasma concentrations and urinary excretion of quinine

The *in vivo* disintegration time of the coated preparations increased with increasing thickness of the coating layer The rate of absorption and the absorbed amount decreased with increasing *in vivo* disintegration time. At a disintegration time of 5 hours the absorbed amount was found to be approximately 60 % of the amount absorbed from uncoated tablets although a high individual variation was found.

Results from investigations of the absorption of quinine HCl from rat intestine are discussed.

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Reunanen M (Department of Physiology University of Turku Finland)
EFFECT OF SALICYLATES AND CINCHOPHEN DERIVATIVES ON AMINO ACID INCORPORATION IN TISSUE SLICES

The ulcerogenic drug cinchophen lowers the secretion of gastroduodenal mucus (Hartiala Ivy and Grossman 1950) Salicylic acid also is known to produce gastric haemorrhages

Cinchophen and its 3 hydroxy derivative as well as salicylic acid asetyl salicylic acid and salicylaldehyde have been shown to inhibit the translation step in protein biosynthesis while other hydroxy derivatives of cinchophen and also salicylamide showed only a slight or no effect (Reunanen Hanninen and Hartiala 1966)

The present study was carried out with rat tissue slices incubated in Krebs Ringer solution containin the C-14 labelled amino acids The TCA insoluble material was counted for radioactivity The addition of 3-hydroxy cinchophen or salicylaldehyde *in the incubation mixture* inhibited the incorporation more than other hydroxy derivatives of cinchophen while *of these drugs salicylic acid acetyl salicylic acid* and salicylamide had the least effect

Cinchophen feeding to rats was shown to decrease the incorporation of amino acids in tissue slices *in vitro*

These results will be discussed in terms of the drug influence on the amino acid penetration into the cells and on the specific activity of the labelled *amino* acid *intracellularly* as well as *the drug intervention with* the protein synthetizing machinery

Hartiala K A I Ivy and M I Grossman, *Amer J Physiol* 1950 *162* 110
Reunanen M O Hanninen and K Hartiala FEBS Third *Meeting* 1966 Abstract F 55

Riekkinen P J T O Ekfors and V K Hopsu (Department of Anatomy University of Turku Finland) ALKALINE TRYPSIN LIKE ENZYMES IN THE RAT SUBMANDIBULAR GLAND AND SALIVA.

The following alkaline trypsin like proteases have been purified from the rat submandibular tissue *salivain* (Riekkinen and Hopsu 1965 Riekkinen *et al* 1966 a) pH optimum at 9.2 hydrolyses synthetic trypsin substrates and proteins haemoglobin faster than casein *glandulain* (Riekkinen *et al* 1966 b) pH optimum at 8.2 hydrolyses the same substrates as salivain but casein faster than haemoglobin a *sulphhydryl-dependent protease* (Riekkinen and Hopsu 1965) pH optimum at 7.0 hydrolyses the same substrates as the former ones four isozymic forms of a *kallikrein-like* (Ekfors *et al* 1966) enzyme pH optimum at 8.2—8.3 hydrolyses synthetic trypsin substrates slowly casein and haemoglobin.

Studies on the relations of the above enzymic activities to various secretory stages of the submandibular gland reveal that only the first mentioned activity is markedly secreted in the saliva Chemical and immunoelectrophoretic studies on the saliva collected from the mouth and the stomach of the rat confirm this finding

Ekfors T O P J Riekkinen T Malmiharju and V K Hopsu *Biochim biophys Acta (Amst)* 1966 In press
Riekkinen P J and V K Hopsu *Ann Med exp Fenn* 1965 43 6—14
Riekkinen P J T O Ekfors and V K Hopsu *Biochim biophys Acta (Amst)* 1966 In press

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Rosell S (Department of Pharmacology Karolinska Institutet Stockholm Sweden) *RELEASE OF FREE FATTY ACIDS (FFA) FROM ADIPOSE* TISSUE DURING SYMPATHETIC STIMULATION

Subcutaneous adipose tissue situated in an area limited by the abdominal midline the pubis and the inguinal region was isolated from the skin and the muscles in anaesthetized dogs The nervous supply one artery and one vein were left intact The adipose tissue was perfused via artery at a constant flow rate with defibrinated blood Samples of arterial perfusion blood and venous blood collected during 1 to 10 min intervals in centrifuge tubes were saved for later analysis of plasma FFA concentration (Dole 1956 Trout Estes and Friedberg 1960) The net uptake or net release of FFA was calculated as the arteriovenous FFA difference times the plasma flow

Electrical stimulation of either the sympathetic trunk at the level of L1 or the distal end of the transected nerve to the adipose tissue produced an increased rate of FFA release It was found necessary to extend the stimulation period to 2 min or more at frequencies between 1—10/s in order to produce an increase in the FFA release Maximal release rate occurred at a stimulus frequency of 3/s (1.5 μEq/min/100 g adipose tissue) At higher frequencies the release rate tended to be lower This may be related to the concomitantly occurring circulatory responses including vasoconstriction which follow sympathetic stimulation Sympatholytic agents with α receptor blocking properties potentiated the FFA release due to sympathetic nerve stimulation whereas β receptor blocking agents inhibited the release

Dole V P *J clin Invest* 1956.35 150
Trout D L E H Ester Jr and F J Friedberg *J Lipid Res* 1960.1 199

XII Scandinavian Congress of Physiology Turku 1966
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Rosenhamer G (Laboratory of Aviation Medicine Department of Physiology Karolinska Institutet Stockholm Sweden) EFFECTS OF HEADWARD ACCELERATION ON CARDIAC OUTPUT AT REST AND DURING EXERCISE

The effects of increased gravitational stress (+3 G) on cardiac output heart rate and oxygen uptake at rest and during leg exercise were measured in eight healthy young subjects in the seated position. Both at rest and during exercise (300 and 600 kpm/min) the cardiac output and stroke volume were found to be significantly reduced ($P < 0.05$) when compared with data at +1 G while the oxygen uptake and AV-O_2 difference were significantly higher ($P < 0.01$) The heart rate and oxygen uptake values at +3 G indicated that relative steady state conditions were kpm/min With the steady state work load of 300 kpm/min the stroke volumes at +1 G and +3 G increased by 21 and 87 per cent respectively over those at rest at the corresponding G-levels The simultaneous increases in heart rate were 51 and 11 per cent

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Rosenkilde P Laboratory of Zoophysiology A University of Copenhagen Denmark) THYROXINE INHIBITION OF IODINE UPTAKE IN THE *THYROIDS OF NORMAL AND HYPOPHYSECTOMIZED TOADS BUFO* BUFO (L.)

It has been shown previously that uptake of I—131 in the thyroid of the toad Bufo bufo is decreased by thyroxine injections to values below those found after hypophysectomy (Rosenkilde 1964) As the amounts of thyroxine used were rather high it was felt desirable to repeat the experiments with graded doses

Intact toads and toads with the pars distalis of the hypophysis removed were injected daily with varying doses of l thyroxine I—131 was given after three days of hormone treatment and the uptake measured after a further five days In intact toads injections of 1 μg thyroxine per day caused a significant decrease in thyroidal iodine uptake Higher doses resulted in a more pronounced inhibition The iodine uptakes of hypophysectomized animals were less than half the normal value and were not further decreased with thyroxine in doses up to 8 μg per day while 50 μg/day gave a significant decrease

It is concluded that thyroxine can inhibit the toad thyroid directly but that the required amounts are too high for a direct feedback control to be of significance in the normal control of thyroid function

Rosenkilde P Regulation of thyroid function in the toad Bufo bufo *Gen comp Endocr* 1964 4 74—81

Rugstad H E. (Institute of Physiology, University of Oslo Norway)
INACTIVATION OF PLASMA KININS BY BACTERIA

The plasma kinins produce the four cardinal signs of inflammation and it has been suggested that they may act as chemical mediators in the inflammatory response

In the present study the following bacteria have been examined for kinin forming and kinin inactivating activity Pseudomonas aeruginosa Escherichia coli and Providencia The microbes were allowed to grow in glucose broth under constant shaking and the cultures were centrifuged at 10000 x g for 15 min Supernatant fluid washed microbes and ultrasonically treated microbes were investigated separately

Kinin forming activity could not be revealed in any of the bacterial species investigated Kinin inactivating activity (kininase) was found in ultrasonically treated bacteria of all the species investigated but not in washed sediments of the microbes (Amundsen and Rugstad 1965) In cultures of Pseudomonas aeruginosa but not in those of the other bacteria marked kininase activity (up to 500 times that of human plasma) was found in the supernatant fluid The kininase produced by Pseudomonas aeruginosa has been studied in detail The kininase is produced also in media composed of inorganic salts and glucose only The production is more marked however in media which give better growth The amount of kininase in the medium is maximal when the curve of bacterial growth has reached the top

The kininase has been purified from cultures of Pseudomonas aeruginosa in protein free medium The purification steps are alcohol precipitation gel filtration and ion-exchange chromatography The final product gives a single band on disc electrophoresis at pH 9,5 and pH 4,3 Studies on the action of this enzyme and of the influence upon its activity of various enzyme inhibitors have been carried out

Amundsen E and H E Rugstad *Brit J Pharmacol* 1965.27 67—73

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Rune S J and F W Henriksen. (Institute of Experimental Research in Surgery University of Copenhagen and Department of Clinical Physiology Bispebjerg Hospital Copenhagen Denmark) GASTRIC ACID OUTPUT AND PANCREATIC BICARBONATE SECRETION FOLLOWING FOOD INGESTION IN THE DOG

Measurements of the acid base balance in multiple arterial blood samples make calculation possible of the net acid loss in the body following stimulation of the gastric acid secretion (Rune 1966) In dogs the rate of net acid loss was measured after feeding a solid meal and the pancreatic juice was collected quantitatively by means of a catheter placed in the pancreatic duct through a duodenostomy opposite the papilla Vateri

The rate of gastric acid secretion in the early postprandial period can be expressed by the rate of net acid loss in the body + the rate of pancreatic bicarbonate secretion + the rate of base elimination in urine

In this way the gastric secretion after solid food was found to be of equal intensity as during a maximal stimulation with histamine The rate of pancreatic bicarbonate secretion in the first hour after the meal was equivalent to 15—20 % of the acid secretion

Rune S J *Gut* 1966 In press

Salmi H A (Department of Physiology University of Turku Finland)
ABSORPTION OF RADIOACTIVE CYANOCOBALAMIN FROM THE DEVELOPING DIGESTIVE CANAL

The development of the absorption of radioactive cyanocobalamin (vitamin B_{12} ^{58}Co) was studied *in vivo* in growing guinea pigs 62 animals 26 in the intrauterine stage (weight range from 3 to 93 grams) and 36 in the postnatal period (weight range from 66 to 310 grams) were used In various stages of pregnancy one foetus in each dam was exposed A thin plastic catheter was introduced into the stomach of the foetus Radiovitamin B_{12} ^{58}Co (dose 0 1 μg/kg) was then injected into the stomach and the mouth of the foetus was closed with a silk string The uterine and abdominal walls of the mother were closed for a period of 24 hours after which the maternal animal was sacrificed and the foetus removed for analysis

In the post partum material the growing animals received the corresponding intragastric feeding of radiovitamin and were killed 24 hours later

The following tissues were analysed for radioactivity the liver the kidney the cardiac muscle the lungs and the carcass The total radioactivity absorbed from the intestinal canal to the abovementioned organs and structures was calculated and plotted against the total body weight representing the developmental stage of the animal

No increase in the absorptive capacity of the digestive canal was found during the development The total absorption remained fairly constant during the whole period studied

Boass and Wilson (1963) suggested two different mechanisms for cyanocobalamin uptake in young rats based on *in vitro* experiments with isolated rat tissues one requiring the presence of intrinsic factor whereas the other is IF independent When little or no IF is produced the IF independent mechanism is dominant In some *in vitro* experiments they found the characteristics of B_{12} uptake by young guinea pig intestine to differ from those of the rat The guinea pig foetuses were not investigated in their study The present *in vivo* results are in agreement with their *in vitro* experiments with rats

Boass A and T H Wilson Development of mechanisms for intestinal absorption of vitamin B_{12} in growing rats *Amer J Physiol* 1963.204 101—104

XII Scandinavian Congress of Physiology Turku 1966
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Saltin B and L Hermansen* (Department of Physiology Gymnastiska Central Institutet Stockholm Sweden) BODY TEMPERATURE DURING EXERCISE AT DIFFERENT PER CENT OF MAXIMAL OXYGEN UPTAKE

It is well known that the central or core temperature rises gradually during the first hour of work to a new level which is proportional to the work load This adjustment of the core temperature is over a wide range (5—30 C) independent of the environmental temperature

Little attention is however paid to the question of the increase in body temperature in relation to the relative work load (*i e* oxygen uptake expressed in per cent of the maximal oxygen uptake)

Rectal esophageal and muscle temperatures were studied in 2 female and 5 male subjects at 25 % 50 % and 70 % of their individual maximal oxygen uptake There is a straight line relationship between the temperature at the end of one hour of work and the oxygen uptake expressed as per cent of the maximal oxygen uptake

According to Benzinger (1960 1963) the sweat intensity is regulated by the hypothalamic and the average skin temperatures

Average skin temperature (besides rectal esophageal and muscle temperatures) and weight loss were measured in a complementary study on two subjects one with a high and the other with a low maximal oxygen uptake both having the same surface area They were both working in the same room for one hour at the same relative work load and both of them ended up with the same rectal esophageal and average skin temperatures but the sweating rate was different Weight loss being 825 g in the fit subject and 500 g in the unfit subject

These results shows that the core temperature is set according to the relative work load of the individuals and not to the absolute work load This study also shows that the fit subject is sweating more than the unfit at the same hypothalamic and average skin temperatures which indicates a lower threshold for eliciting sweating in the fit subject

* On leave from the Institute of Work Physiology Oslo Norway

Samuelsson R. and U Sjostrand (Institute of Physiology and Medical Biophysics University of Uppsala Sweden) INTRACARDIAC RECORDINGS OF MONOPHASIC ACTION POTENTIALS IN THE DOG HEART IN SITU

A technique for intracardiac recording of monophasic action potentials has been developed (Sjöstrand 1966) and some results are presented The method appears to provide the requirements for extended studies of excitation rhythmicity and impulse propagation in the heart in situ in both man (Korsgren Leskinen Sjostrand and Varnauskas 1966) and lower animals and gives recordings which can be correlated in time with the conventionally recorded electrocardiogram The method allows recording of the electrical activity in subendocardially situated atrial and ventricular muscle as well as in subendocardially situated parts of the conducting system with further development of this method it should be possible to study changes in the amplitude form and duration of the action potential

Korsgren M E Leskinen U Sjostrand and E Varnauskas Intracardiac recording of monophasic action potentials in the human heart *Scand J clin Lab Invest* 1966 In press
Sjöstrand U A method for intracardiac recording of monophasic action potentials in the dog heart in situ *Acta physiol scand* 1966 In press

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Secher Hansen E H Langgård and J Schou (Institute of Pharmacology University of Copenhagen Denmark) OEDEMA FORMATION CAUSED BY SUBCUTANEOUS INJECTIONS IN MICE

In a series of investigations on the importance of the connective tissue ground substance in subcutaneous absorption of water and water soluble substances the local oedema formation caused by the subcutaneous injection *per se* has been examined

The method used permits the simultaneous registration of the uptake by a well defined skin area of circulating radioiodionated albumin (RISA) and the total contents of biogenic amines in the same skin sample

The accumulation of radioactivity has been examined after subcutaneous injection of one of the following solutions 0 9 % NaCl 10 % sucrose 14 % sucrose and water to untreated or oestradiol treated mice In parallel groups of animals the same solutions were injected containing hyaluronidase 500 units/ml

The results indicate that the trauma of the injection causes the extravasation of significant amounts of plasma largely proportional to the size of the volume injected The degree of oedema formation however was independent of the nature of the injected solutions and of the condition of the connective tissue ground substance The injections did not result in measureable losses of histamine or serotonin from the injection sites

Sejrsen P (Department of Clinical Physiology Bispebjerg Hospital Copenhagen Denmark) THE EPIDERMAL DIFFUSION BARRIER TO INERT GAS STUDIED BY XENON 133 IN MAN

Development of a method for measuring cutaneous blood flow by clearance of the radioactive inert gas Xenon 133 raised the question of a loss by diffusion of Xenon 133 out through the epidermis One of the conditions of using an isotope clearance method for estimating values of cutaneous blood flow is that the isotope is cleared only by the blood

Xenon 133 dissolved in an isotonic saline solution was injected intracutaneously into the crus in man A tourniquet arresting the circulation was placed proximal to the knee just before the injection By external monitoring of the γ-radiation the loss of Xenon 133 out through the epidermis was followed in 12 to 15 minutes and expressed as the time constant for the measuring results plotted in a semilogarithmic diagram Similar measurements were performed after the removal of the epidermal cells by successive strippings with adhesive plaster for every ten times of stripping to a maximal of 50 times The exposed layers in the epidermis were localized by microscopic examination of cell specimens removed by stripping with adhesive tape and stained by the May—Grunwald—Giemsa technique

A diffusion barrier to Xenon 133 in the epidermis was found and the regeneration of the barrier after removing by stripping was followed The loss of Xenon 133 through the intact epidermis was estimated to be negligible in relation to the clearance by blood flow The possibility of a considerable loss of Xenon 133 by sweating was demonstrated

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Siirtola T O and A Pekkarinen (Department of Pharmacology University of Turku Finland) A STUDY ON RED CELL AND PLASMA ACHE ACTIVITIES OF FINNISH FARMERS AND INDUSTRIAL WORKERS

The acetylcholine esterase activities of red cell and plasma in 42 farmers and 41 industrial workers handling parathion insecticides and in 46 healthy control persons were measured by Michel s electrometric method in some persons up to 11 months

Normal values of the control group were 0 83 Δ pH/h for red cell and 0 89 Δ pH/h for plasma in men and 0 83 Δ pH/h for red cell and 0 68 Δ pH/h for plasma in women

One red cell and five plasma AchE activities in farmers are below 70 % of mean control values (Red cell 69 % and plasmas 39 % 57 % 62 % 64 % and 64 %) All of these farmers had handled parathion insecticides recently but no one had subjective symptoms

Three red cell and eight plasma AchE activities in industrial workers are below 70 % of control value (lowest red cell value 53 % and plasma 40.5 %) All these values were controlled up to 10—11 months Two red cell and three plasma AchE values are lower than normal range of individual daily variation All the values were slowly normalized during the observation period Some of the industrial workers complained of headache and nausea

Our results indicate the need of close control of AchE activities in industrial workers handling parathion and also more effective control of the use of parathion on farms

In Finland however the most important problem of insecticide control is the control of the use and storage of insecticides by more strict legislation and better labelling of packages especially for parathion to prevent the high mortality rate with parathion The number of suicides and deaths has decreased clearly from the maximum 128 in 1958 but only to 43 and 39 in 1963—64 which are also far too high

Skadhauge E. and B Schmidt Nielsen (Department of Biology Western Reserve University Cleveland Ohio U.S.A. and Institute of Medical Physiology A University of Copenhagen Denmark) THE RENAL MEDULLARY ELECTROLYTE AND UREA GRADIENT IN ROOSTERS AND TURKEYS

Bird kidneys are supposed to function as countercurrent systems like mammalian kidneys This enables the kidney to produce a urine with a higher osmotic concentration than the blood In mammals the chief end product of nitrogen metabolism urea may account for up to fifty per cent of the papillary hypertonicity but in birds the majority of nitrogen is not excreted as urea but as uric acid

The composition of the renal medulla was investigated in roosters and turkeys exposed to dehydration and to water and salt loading Renal medullary tissue was obtained by micro dissection of the frozen kidney Sodium chloride and urea was found in a higher concentration in the medulla than in the cortex during dehydration and salt loading but the sodium chloride accounted for the majority of the hypertonicity and urea contributed less than one half per cent to the total osmolality due to the low absolute concentration

In turkeys sufficiently large pieces of medullary tissue could be dissected in order to demonstrate an intramedullary gradient for sodium chloride and urea

During hydration the osmotic (sodium chloride) gradient was reduced.

It was also observed that all filtered urea was excreted in the urine during hydration but less than one per cent during dehydration

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Skou J C (Institute of Physiology University of Aarhus Denmark)
RELATIONSHIP OF Na + K ACTIVATED ENZYME SYSTEM TO THE ACTIVE TRANSPORT OF CATIONS

The Na + K activated ATP hydrolyzing enzyme system contains two sites with specific affinities for monovalent cations one where the affinity for Na is higher than for K and another where the affinity for K is higher than for Na Evidence is given that these sites can change their affinities for Na and K and that this is due to an effect of ATP on the enzyme system *The two sites thus seem to fulfil the requirements to carrier sites* This lends further support to the view that this enzyme system is involved in the active transport of Na and K across the cell membrane and may suggest that the enzyme system represents the transport system

Solatunturi E and M K Paasonen (Department of Pharmacology University of Helsinki Finland) SUBCELLULAR DISTRIBUTION OF MONOAMINE OXIDASE (MAO) 5-HYDROXYTRYPTAMINE (5HT) AND HISTAMINE IN BLOOD PLATELETS OF RABBIT

It is known that platelet 5HT is stored in subcellular particles (Baker *et al* 1959 Buckingham and Maynert 1964 Wurtzel *et al* 1965)

Platelets were homogenized in 0.32 M sucrose by using ultra sound (Branson Sonifier S-75 with 1/8 micro tip setting No 3 1 min) The homogenate was fractionated by differential centrifugation according to Buckingham and Maynert The MAO 5HT and histamine contents of the fractions are presented in the following tabulation

Fraction (g×min)	MAO %±SE (7 experiments)	5HT %±SE (5 experiments)	Histamine %±SE (3 experiments)
2 500×20	6.5±1.8	14.3±0.4	12.5±0.8
18 500×30	82.7±6.1	67.5±1.9	62.0±7.2
100 000×60	3.6±1.6	1.7±0.3	1.3±0.2
Supernatant	6.7±3.4	16.6±2.5	24.3±6.8

When the 18 500 g sediment was resuspended and centrifuged over a discontinuous density gradient (100 000 g 60 min) five subfractions were obtained The fraction with a density similar to about 1.4 M sucrose contained most of MAO and the fraction heavier than or equal to 2.0 M sucrose most of 5HT and histamine (Supported by USPHS Grant MH 5832)

Baker R V H Blaschko and G V R Born *J Physiol (Lond)* 1959 *149* 55P—56P
Buckingham S and E W Maynert *J Pharmacol exp Ther* 1964 *143* 332—339
Wurtzel M A J Marcus and B W Zweifach *Proc Soc exp Biol (NY)* 1965 *118* 468—472

XII Scandinavian Congress of Physiology Turku 1966
Acta physiol scand 1966.68 Suppl 277

Squires R and J B Lassen (Research Laboratories of Ferrosan Copen hagen Denmark) SOME PHARMACOLOGICAL AND BIOCHEMICAL PROPERTIES OF γ-MORPHOLINOBUTYROPHENONE (NSD 2023) A NEW INHIBITOR OF MONO AMINE OXIDASE

Pharmacological experiments on rats and mice have shown that NSD 2023 has a weak sedative effect of very short duration and a more pronounced anticonvulsant effect also of short duration In tests for reserpine antagonism (ability to reverse reserpine induced hypothermia and ptosis) and MAO inhibition *in vivo* the substance showed a pronounced effect of longer duration

Maximum inhibition of mouse brain MAO *in vivo* occurs within 5 minutes of oral administration Duration of inhibition is dose dependent After 50 mg/kg orally about 7 or 8 hours are required for 50 % regeneration of inhibited enzyme Half maximum inhibition is obtained 1—2 mg/kg orally when mice are killed one hour after administration

Complete inhibition of MAO by NSD 2023 has never been observed either *in vivo* or *in vitro* Instead a limiting value for inhibition (less than 100 %) is approached asymptotically with increasing NSD 2023 concentration *In vivo* maximum inhibition is between 60 and 80 % In vitro inhibition is generally less Greatest inhibition by NSD 2023 *in vitro* is observed in whole organ homogenates Washed liver mitochondria are relatively insensitive to inhibition by NSD 2023

Pre treatment of mice with SKF 525 A does not appear to have any effect on *in vivo* inhibition of MAO by NSD 2023 It is tentatively concluded that the MAO inhibition observed both *in vivo* and *in vitro* is produced by NSD 2023 itself and not by one of its metabolites

Pre treatment of mice with NSD 2023 protects to some extent brain MAO from irreversible inhibition by phenelzine pargyline tranylcypromine iproniazid and isocarboxazid Pre-treatment of mice with harmaline protects brain MAO from inhibition by NSD 2023

NSD 2023 a non-competitive inhibitor of MAO using tryptamine as substrate NSD 2023 inhibition of MAO is irreversible in the sense that inhibition cannot be reversed by dilution or by washing mitochondria

NSD 2023 produces an increase in the cerebral levels of serotonin and noradrenaline and retards the depletion of these amines induced by reserpine

MAO inhibition by NSD 2023 is not oxygen dependent Inhibition in vivo does not appear to be greatly influenced by route of administration

In brain and kidney MAO is inhibited more than in liver

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Solatunturi E. and M K. Paasonen (Department of Pharmacology University of Helsinki Finland) SUBCELLULAR DISTRIBUTION OF MONOAMINE OXIDASE (MAO) 5-HYDROXYTRYPTAMINE (5HT) AND HISTAMINE IN BLOOD PLATELETS OF RABBIT

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18 500×30	82 7±6 1	67.5±1 9	62 0±7.2
100 000×60	3 6±1 6	1 7±0.3	1.3±0.2
Supernatant	6 7±3 4	16 6±2.5	24 3±6 8

When the 18 500 g sediment was resuspended and centrifuged over a discontinuous density gradient (100 000 g 60 min) five subfractions were obtained The fraction with a density similar to about 1 4 M sucrose contained most of MAO and the fraction heavier than or equal to 2 0 M sucrose most of 5HT and histamine (Supported by USPHS Grant MH 5832)

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Wurtzel M A J Marcus and B W Zweifach *Proc Soc exp Biol (N Y)* 1965 *118* 468—472

XII Scandinavian Congress of Physiology Turku 1966
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Stripp B and E Secher Hansen (Department of Pharmacology Danmarks Farmaceutiske Højskole Copenhagen Denmark) AN OPERATIVE METHOD FOR TOTAL EXCLUSION OF THE LIVER CIRCULATION IN THE RAT

The liver is shunted from the blood circulation by ligating v cava proximally and distally to the liver round a polyethylene tube previously placed in v cava through v iliaca The muzzle of the polyethylene tube is placed directly under the right atrium A cannula is placed in v portae from which the blood drips into a beaker at a rate of 1 to 1 5 ml/min From this beaker which beforehand contains 8 ml of heparinized blood from a donor rat the blood is transferred by a peristaltic pump into the polyethylene tube in v cava The pump is essential for maintaining a blood pressure of 40 to 50 mm Hg for a period of 2—4 hours

A control with a bromsulphthalein test has shown a complete elimination of the liver circulation after the operation

Suihkonen, J and N Raiha (Department of Medical Chemistry University of Helsinki Finland) DEVELOPMENT OF ENZYMES FOR UREA BIOSYNTHESIS IN RAT LIVER

It has been shown previously using liver slices that the capacity to synthesize urea developes soon after birth in the rat This development can be blocked by actinomycin D and puromycin indicating that DNA directed protein synthesis is necessary The development of the overall urea synthesizing capacity in liver slices has been correlated to the development of the individual enzymes of the urea cycle The development of arginine synthetase correlates best with the urea producing capacity of liver slices suggesting that this forms the rate limiting step in urea biosynthesis

The postnatal increase of arginine synthetase activity can be blocked by puromycin and by adrenalectomy

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Sundquist H., A Pekkarinen and K Manninen (Department of Pharmacology University of Turku Finland) INHIBITION OF NICOTINE HYPERTENSION BY NEUROLEPTIC AND ANTIHYPERTENSIVE DRUGS

In the experimental nicotine hypertension the blood pressure increases to its maximum in 4—5 months confirming the results of Wenzel *et al* (1964) and decreases thereafter slowly to the normal blood pressure or even to under the normal Nicotine was given to rats with drinking water (average dose 1 7 mg/kg/24 hrs) Drugs were administered either in drinking water or in food Nicotine treatment increased the blood pressure in 14 weeks from 113 to 146 mm Hg and then the blood pressure decreased slowly in 7 months to 128 mm Hg In other groups the blood pressure rose to its maximum in 14—17 weeks except with thioridazine in 20 weeks and with opipramol in 12 weeks

Reserpine α methyldopa (50 mg/kg/24 hrs) ($p<0\,01$) as antihypertensive drugs phentolamine as sympatholytic ($p<0\,01$) and cloroprothixene as a neuroleptic drug prevented significantly ($p<0\,001$) the development of nicotine hypertension The most effective inhibitors of nicotine hypertension were phentolamine (12 mg/kg/24 hrs) and reserpine (3 mg/kg/24 hrs) The maximum of blood pressure increase during reserpine or phentolamine treatment was only 33 % of nicotine hypertension without these drugs during methyldopa treatment 49 % and guanethidine (15 mg/kg/24 hrs) treatment 69 % A small dose of reserpine (0.5 mg/kg) combined with Hygroton (10 mg/kg) could not inhibit the nicotine hypertension

Among neuroleptic drugs the most effective inhibitor of nicotine hypertension was chlorprothixene (10 mg/kg/24 hrs) The maximum increase of blood pressure was during chlorprothixene treatment 45 % from that of nicotine hypertension without this drug during opipramol ($p<0\,01$) (20 mg/kg/24 hrs) 57 % chlorpromazine ($p<0\,05$) (10 mg/kg/24 hrs) 61 % imipramine ($p<0\,01$) (18 mg/kg/24 hrs) 64 % thioridazine ($p<0\,01$) (25 mg/kg/24 hrs) 67 % and amitriptyline ($p<0\,01$) (10 mg/kg/24 hrs) 76 % In one series also chlorprothixene (10 mg/kg) was not effective

Wenzel D G A Wattanapongsiri and D Vedral *J Pharmacol exp Ther* 1964.145.315

Suihkonen J and N Raiha (Department of Medical Chemistry University of Helsinki Finland) DEVELOPMENT OF ENZYMES FOR UREA BIOSYNTHESIS IN RAT LIVER

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The postnatal increase of arginine synthetase activity can be blocked by puromycin and by adrenalectomy

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Suramo I S Saarikoski and A Pekkarinen (Department of Pharmacology University of Turku Finland) THE EFFECT OF IRREVERSIBLE HAEMORRHAGIC SHOCK ON THE FLUORESCENCE REACTION OF ADRENERGIC NERVE FIBRES AND THE CONTENT OF NOR ADRENALINE IN THE HEART SPLEEN AND KIDNEY OF RABBITS

An experimental irreversible hemorrhagic shock was produced in 9 rabbits (8 controls) during pentobarbitone (25—30 mg/kg) and lidocaine anesthesia Through a canule in the femoral artery blood was bled into an elevated transfusion reservoir (about 30 cc/kg) until the blood pressure was stabilized at 50 mmHg (in 6 experimental series) The hypotension continued for 10—19 hours before the heart spleen and kidney were taken for histochemical examination according to Falck Owman and Hillarp (1965) and for the chemical determination of noradrenaline and adrenaline with v Euler's and Flodin s trihydroxyindole method (1956) modified by Pekkarinen Freeze-drying was made in a copperplate chamber in a —35 C ethanol bath connected to a cold compressor and a vacuum pump for 2—3 days

In the heart of the control rabbits the fluorescence reaction of the adrenergic nerve fibres of the wall of the left ventricle was intensive and clearly reduced during the hemorrhagic shock In the spleen the green fluorescence reaction of adrenergic fibres of the control rabbits was diminished by the shock The fluorescence reaction of the adrenergic nerve fibres in the renal cortex of the control rabbits was intensive in the walls of blood vessels and capillaries outside the glomeruli but not inside them the fluorescence reaction of both the afferent and the efferent extra glomerular arterioles was often intensive During the shock the adrenergic nerve fibres of the renal blood vessels and those of both the afferent and the efferent extraglomerular arteries were weaker and thinner and often their fluorescence reaction was strongly decreased

The mean noradrenaline content of the heart decreased during the shock to 47 1 % in the spleen to 36.2 % of the normal value while the mean noradrenaline content in the kidney generally was at normal level (101 %) Since the fluorescence reaction of adrenergic nerve fibres of the kidney showed a clear reduction in shock while the noradrenaline content of kidney was normal it seems that in shock noradrenaline mainly appears there extraneuronally

v Euler U S *Noradrenaline* 1956 p 90 Charles C Thomas Springfield U.S.A 1956 p 90

Falck B and Owman C *Acta Univ Lundensis Sect II 1965* No 7 C W.K Gleerup Lund

Sutherland G R and O Rasanen (Department of Radiology University Central Hospital Turku Finland) EFFECT OF RADIOGRAPHIC CONTRAST AGENTS ON THE VASCULAR BED OF THE RAT MESENTERY *

The Radiographic contrast agents cause crenation and sludging of the red blood cells and damage to the vascular walls of small blood vessels This causes leucocyte adhesion to the vessel wall There are certain differences between various contrast agents

* A film made using intravital microscopy

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Takki S M M Airaksinen and M J Mattila (Department of Pharmacology University of Helsinki Finland) STUDIES ON TOXICITY OF FERN EXTRACTS AND ABSORPTION AND METABOLISM OF THEIR PHLOROBUTYROPHENONE DERIVATIVES

Oral toxicity of desaspidin flavaspidic acid as well as fern extracts of different origin were studied in mice Desaspidin was twice as toxic as flavaspidic acid Considerable differences were found in the toxicity of different fern extracts and chromatographically also in the content of different phlorobutyrophenones Extractum dryopteridis austriacae was nearly twice as toxic as Extractum filicis

The uptake of desaspidin by *Diphyllobothrium latum* and the intestine of its host dog were compared *in vitro* Uptake of the anthelmintic by the parasite and the host intestine was about the same magnitude However the biotransformation of desaspidin was confirmed by chromatographic methods which revealed a formation of several other phlorobutyrophenone derivatives durings the incubation of the dog s intestine but much less during that of the tapeworm Thus the selective anthelmintic toxicity seems to depend on the lower metabolism of drugs in the tapeworm than in the host

Teorell T (Institute of Physiology and Medical Biophysics Uppsala Sweden) ELECTROKINETIC CONSIDERATIONS OF A MECHANO ELECTRICAL TRANSDUCTION

An electrokinetic mechano-electrical transducer analogue has been subject to detailed analysis It consists of a dual-compartment model of a pressure sensitive cell bounded by a membrane which can be represented as a micro-porous elastic structure containing fixed charge groups This ion and water permeable membrane is polarized by a constant electric current The model is metastable in the sense that it can be stimulated both by changes of the electric current and/or by changes of a pressure gradient It has been shown that the artificial transducer elicits a static response to maintained constant pressure stimuli in the form of repetitive action potentials The relation impulse frequency versus stimulus strength is largely linear and analogous to many of those reported in the physiological literature on pressure receptors The model shows also a dynamic type of responses With the background of existing concepts in receptor physiology a hypothetical 1-step process (direct coupling between pressure effect and electrical response) and a 2 step process (involving a primary generator potential) will be discussed The role of the polarizing electric resting current is considered as being a manifestation of the existence of a biochemically driven fuel cell

Teorell T Some biophysical considerations on presso-receptors *Arch Int Pharmacodyn* 1962 140.563—576
Teorell T Electrokinetic considerations of a mechano-electrical transduction *Ann N Y Acad Sci* 1966 In press

XII Scandinavian Congress of Physiology Turku 1966
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Terho T and K Hartiala (Department of Physiology University of Turku Finland) **INCORPORATION OF $^{35}SO_4^{=}$ INTO SUBMICROSOMAL FRACTIONS ISOLATED FROM RAT LIVER AND INTESTINE**

The incorporation of $^{35}SO_4^{=}$ into subcellular components known to be implicated in protein synthesis was investigated $Na_2{}^{35}SO_4^{=}$ was injected intraperitoneally into rats and the submicrosomal fractions were isolated according to Kornfeld *et al* (1965)

In intestine the deoxycholate soluble (membranous) fraction of microsomes proved to contain much more $^{35}SO_4^{=}$ than the ribosomes In liver the situation was reversal into ribosomes more $^{35}SO_4^{=}$ was incorporated than into membranes The ribosomal membranes isolated from intestine were labelled more highly than the membranes of liver A little more radioactivity was incorporated into ribosomes of liver than into ribosomes of intestine The maximal incorporation was found 1.5—2.5 hours after injection

$^{35}SO_4^{=}$ has been used in this work as an indicator of synthesis of sulphomucopolysaccharides The results indicate that the intracellular site of sulphation of mucopolysaccharides is the membraneous fraction of microsomes and the sulphation and evidently also the biosynthesis of mucopolysaccharides are much more strongly increased in intestine than in liver Extraction of microsomes with deoxycholate released compounds which were very highly labelled The radioactivity of these compounds was greater in intestine than in liver It is possible that this material contains mainly macromolecules which are just under synthesis It may be possible that $^{35}SO_4^{=}$ accumulated into ribosomes of liver and intestine will be used for biosynthesis of sulphate containing proteins

Kornfeld S R Kornfeld and V Ginsburg *Arch Biochem Biophys 1965* *1* 110

Supported by U S Public Health Service Grant No AM 6018

Thorn N A (Institute of Medical Physiology A University of Copenhagen Denmark) IN VITRO STUDIES OF THE RELEASE MECHANISM FOR VASOPRESSIN

Recent findings have indicated that calcium is crucially involved in the mechanism of release of the neurohypophysial hormones The details of the processes for which calcium is essential have not been clarified but it has been suggested (Thorn 1965) that calcium ions act by detaching hormone from binding to neurophysin the carrier protein thus allowing the octapeptide to diffuse into the blood stream It would be simple to assume that this process occurs in the axoplasm that calcium ions enter there on depolarization and that the octapeptide diffuses through the nerve membrane into the blood stream after release from protein binding

In the present experiments isolated posterior pituitary hemilobes from rats released only approximately 11 % of their total hormone content when they were stimulated by depolarization with a high concentration of potassium in the medium No more than this percentage could be released in spite of the application of numerous variations in the stimulation procedure and of other stimuli No release of vasopressin binding protein could be demonstrated

Addition to the medium of caffeine and various substances which are known to cause an *in vivo* release of hormone (acetylcholine nicotine and amyl nitrite) had no effect on the rate of release *in vitro*

The results seem to be in accordance with the hypothesis that secretion of vasopressin involves a release of octapeptide from a small pool of readily available hormone that secretion is initiated by nervous impulses arising in the dendrites of the neurosecretory cells and that no transmitter other than calcium is active in the neural lobe

Thorn N A *Acta endocr (Kbh)* 1965.50 357

XII Scandinavian Congress of Physiology Turku 1966
Acta physiol scand 1966.68 Suppl 277

Tissari A and E. M Pekkarinen (Department of Pharmacology University of Helsinki Helsinki Finland) 5-HYDROXYINDOLEACETIC ACID IN THE DEVELOPING BRAIN

For information on the level of free and active 5-hydroxytryptamine (5-TH) in brain during development we have examined the 5-hydroxyindoleacetic acid (5-HIAA) content in the brain of developing rat rabbit and guinea pig 5-HIAA was determined fluorimetrically by the method of Ashcroft and Sharman (1962) in brain stem and hemispheres and after the extraction of the 5-HIAA 5-HT was assayed from the same samples fluorimetrically

In one-day-old rats the 5-HIAA content of the brain stem was about 90 % and that of the hemispheres 60 % of the adult content while the corresponding 5-HT contents were only about 40 % and 30 % A similar trend between relative brain 5-HIAA and 5-HT levels was also found in foetuses on days 22 and 19 after impregnation on the 22nd day the 5-HIAA content in the brain stem and hemispheres was about 60 % and 35 % and the 5-HT content about 30 % and 20 % of the adult content In one-day old rabbits the relative 5-HIAA level of brain stem and hemispheres was only *slightly higher than their relative 5-HT level the 5-HIAA content* being 77 % and 64 % respectively of the adult content and the 5-HT content in both about 50 % In 1 and 3-week-old rabbits no difference between the relative 5-HIAA and 5-HT levels was any longer detectable In the 1 week-olds the 5-HIAA content of the brain stem was 80 % and that of the hemispheres 70 % and in the 3-week-olds the contents were 113 % and 90 % of the adults A very low 5-HIAA content or none at all was measureable in the brain of adult guinea pigs and full term foetuses After administering 5-hydroxytryptophan (5-HTP) to pregnant guinea pigs however the 5-HIAA content of their brain stem and hemispheres rose to about 1 μg/g and 0.5 μg/g respectively but the content in full term foetuses increased to only about 1/3 of these values and the 5-HT content to about 60 % of the maternal level

Since the distribution of 5-HT between particulate and soluble fraction in new born rat brain is the same as in the adult (Bennett and Giarman 1965) its 5-HIAA level probably reflects the magnitude of functional depletion of 5-HT This seems to be higher than expected from the brain 5-HT store at that time and is consistent with the near adult brain 5-HTP decarboxylase activity and adult 5-HTP uptake (Bennett and Giarman 1965)

Ashcroft G W and D F Sharman Drug induced changes in the concentration of 5-OR indolyl compounds in cerebrospinal fluid and caudate nucleus *Brit J Pharmacol* 1962.19 153—160

Bennett D S and N J Giarman Schedule of appearance of 5-hydroxytryptamine (serotonin) and associated enzymes in the developing rat brain *J Neurochem* 1965.12 911—918

Tonnesen K. H and P Sejrsen (Department A Institute of Medical Physiology University of Copenhagen and Department of Clinical Physiology Bispebjerg Hospital Copenhagen Denmark) **THE MUSCLE BLOOD FLOW CALCULATED FROM THE DESATURATION CURVE OF XENON 133 AND BY DIRECT RECORDING**

Recently Zierler has published equations for calculating tissue blood flow by external monitoring of a radioactive tracer administered by rapid intra arterial injection to the tissue in question Assuming that the arrival of the tracer is sufficiently fast then the ratio of the maximal height of the curve (H) and the total area under the curve (A) equals the reciprocal mean transit time t i e $H/A = 1/t$ This approach neither presupposes homogeneous tissue blood flow nor diffusion equilibrium between tissue and blood for the indicator In order to calculate the blood flow per gram tissue f it is necessary to know the equilibrium volume of distribution of the indicator λ since $f/\lambda = 1/t$ or $f = \lambda \cdot H/A$

This paper concerns an in vivo comparison of blood flow measured by Zierler s approach and by a direct method using a dropcounter device The isotope used was the radioactive gas Xenon 133 which is effectively cleared at the lungs thus keeping tracer recirculation at a neglible level The tissue was the isolated gastrocnemius muscle of the cat studied in the working condition (electric stimulation of the nerve) and in the resting condition Care was taken to remove all visible fat at the entrance of the vessels and to collimate the scintillation probe so that only the muscle tissue was seen This was essential as isotope labelling of the thigh muscle could not in all cases be excluded with the fairly gentle type of preparation used

A good agreement between the two was found

The clearance curves were not monoexponential i e the working muscle differs somewhat from that of an idealized Krogh tissue cylinder within which there is the same probability of clearance for inert gas at all points

The present study represents a continuation of the experiments by Kjellmer *et al* (1966)

Kjellmer I I Lindbjærg I Prirowsky and K H Tonnesen *Acta physiol scand* 1966 In press

Zierler K L *Circulat Res* 1965 16 309

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Ungerstedt U (Department of Histology Karolinska Institutet Stockholm Sweden) EFFECT OF INTRAVENTRICULAR INJECTIONS ON THE CENTRAL MONOAMINE NEURONS

After intraventricular injection of α methylnoradrenaline (1—10 μg/kg) to reserpinized rats an uptake of the amine could be observed in DA and NA nerve terminals non terminal axons and cell bodies when present close to the ventricles at the different time intervals studied (5—120 min)

An uptake could also be observed in the pericytes of the capillary walls and in the extraneuronal space present close to the ventricles The amine was also taken up in those 5-HT neurons that were present close to the ventricles

If the animals had been depleted of their catecholamines with a potent synthesis inhibitor (H⁴⁴/68) an uptake was observed after injection of L noradrenaline without MAO inhibition

The effect of intracerebral injections of NA and α methyl NA on the central monoamine neurons has also been studied

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Ursin H., K. Wester and R. Ursin (Neurophysiological Laboratory Institute of Anatomy University of Oslo Norway) HABITUATION TO ELECTRICAL BRAIN STIMULATION IN UNANESTHETIZED CATS

Electrical stimulation of certain brain areas yields behavioural and electroencephalographical arousal This arousal seems identical to the orienting behaviour displayed by an individual suddenly experiencing a change in the environment (the orienting reflex of Pavlov) If this stimulus is repeated several times and is of no consequence to the subject the arousal response will gradually decrease and finally be totally absent This is called habituation

A total of 35 electrode sites in 15 cats has been tested for habituation to electrical brain stimulation All electrode sites evoked orienting behaviour and EEG arousal initially Stimulations lasted for 5 sec and were repeated irregularly with no less than 30 sec inter trial intervals The rate of habituation depended on the localization of the electrode

The cats habituated slowly or not at all (more than 100 trials) to stimulation through electrode sites in the mesencephalic reticular formation (n=10) Amygdala placements (n=10) gave rise to rapid habituation (6—17 trials) except in one case where the stimulation also yielded defense behaviour (60—70 trials) The cats also habituated to neocortical stimulation (gyrus suprasylvicus posterior n=5 8—53 trials) Medial septal placements gave slow habituation or none at all (more than 100 trials) placements in the neighbouring areas gave rapid habituation

The findings are in agreement with previous hypothesis of amygdala and cortical arousal being due to activation *via* midline arousal producing neurons The slow habituation rate of septal placements may be due to the positively reinforcing effects of such stimulation Dishabituation was produced by other arousal producing stimuli including brain stimulation

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Ursin R (Neurophysiological Laboratory Institute of Anatomy University of Oslo Norway) SLEEP PATTERNS IN THE CAT

From 11 freely moving cats frontal and occipital electrocorticograms (ECoG) neck electromyograms (EMG) and eye movements were recorded Two or three recording sessions each of 24 hours duration were run for each cat The animals behaviour was observed continuously throughout the sessions The length of the various sleep-wakefulness stages was calculated for each 24-hour session The following stages were identified

(a) Waking and drowsiness (ECoG from fast low voltage activity in arousal to bursts of high voltage 4—8/sec waves in drowsiness)

(b) Light sleep (ECoG high voltage 12—14/sec sleep spindles and random slow waves on a low voltage fast background EMG well sustained activity No eye movements)

(c) Deeper sleep (ECoG high voltage 1—4/sec waves and sleep spindles EMG variable but always some activity No eye movements)

(d) Paradoxical sleep (ECoG fast low voltage waves EMG no tonic activity Bursts of rapid eye movements)

The normal pattern of wakefulness and sleep stages is fairly constant for each cat but there are some individual variations The mean value of the total sleep time for the whole group was 57 8 per cent (S D = 8 6) Light sleep constituted 29 7 per cent (S D = 11 1) of total sleep time deeper sleep 47 0 per cent (S D = 9 0) and paradoxical sleep 23 3 per cent (S D = 3 3) The amount of paradoxical sleep was positively correlated to the amount of deeper sleep ($p<0\ 05$) but not to light sleep

This study of normal sleep patterns is part of studies of the effect of brain lesions environmental changes and drugs on sleep

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Uusitalo A. J (Institute of Physiology University of Helsinki Finland)

ALBUMIN *PRODUCTION* BY LIVER SLICES IN *THE DEVELOPING GUINEA PIG*

The albumin production in the liver of guinea pigs at *different* developmental stages was determined 1) by measuring the *incorporation rate of* ^{14}C-*labelled* leucine into the albumin and 2) by *measuring* the quantity *of albumin* produced by liver slices during two hours incubation. The albumin secreted by liver slices was precipitated with a *specific* antiserum *made in* this laboratory (Uusitalo 1966)

During the foetal experimental period albumin production *increased* rapidly *from the* 49th to the 60th foetal day On the 60th foetal day the amount of albumin *produced* by liver slices was about *five times* as great and the radioactivity of the albumin about three times as high as the respective values on the 49th day After the 60th foetal day there was a decrease in albumin production during the last four *prenatal and first five* postnatal days On the 5th postnatal day the values for both albumin quantity and radioactivity were at their lowest After the 5th postnatal day albumin production again increased On the 60th foetal day the *rate of* albumin *production* was at least 100 per cent higher but on the 5th postnatal day about 40 per cent lower per gram of sliced liver than in adult guinea-pigs

The different phases of liver development with accompanying biochemical changes (Uusitalo 1966) are correlated with the changes in the rate of albumin production.

Uusitalo A. J Studies *on the* protein metabolism and related phenomena in the developing liver of the guinea pig. *Ann Acad Sci fenn.* A 5 1966 *126* In press

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Vahvelainen M.L., S S Oja and A J Uusitalo (Institute of Physiology University of Helsinki Finland) FREE AMINO ACIDS IN THE BRAIN AND LIVER OF THE DEVELOPING RAT AND GUINEA PIG

Amino acids were separated by paper chromatography and stained with ninhydrin and their concentrations measured spectrophotometrically The material consisted of foetuses and young animals of different ages and adults

The concentrations of glutamic acid and gamma aminobutyric acid increased in the brain of both species during development In the foetal period the concentration of glutamine increased in all the organs studied but thereafter the changes were not so pronounced The concentration of alanine in the brain was relatively high in the foetal period and decreased during the postnatal period In the liver the changes in the alanine concentration showed no clear trend The changes in the concentration of glycine and serine resembled those of alanine The concentrations of ethanolamine phosphate and taurine decreased postnatally in the rat their concentrations were lower in the foetuses than in the newborn but just the opposite was observed in the guinea pig In the rat the concentration of taurine was many times higher than in the guinea pig The concentrations of some other amino acids differed too but not so significantly The concentrations of individual amino acids were generally more consistent in the brain than in the liver The concentration changes of alanine leucine ethanolamine phosphate glutamic acid gamma aminobutyric acid glycine and serine were similar in both animals especially in the brain Glutamine aspartic acid taurine and glutathione however behaved quite differently in the rat than in the guinea pig

Valleala P (National Institutes of Health Bethesda Md USA and Department of Physiology University of Turku Finland) THE TEMPORAL RELATION OF UNIT DISCHARGE IN VISUAL CORTEX AND PHASIC ACTIVITY OF THE LATERAL RECTUS EYE MUSCLE DURING POSTCALORIC HORIZONTAL NYSTAGMUS

The purpose of this work was to study the visuovestibular integration in the striate cortex of an unanesthetized monkey whose eyes were covered by opaque contact occluders The monkey was sitting in a primate chair and the head was bent backward 60 degrees and stabilized with a special arrangement Following the unilateral 20—23° C calorization the electrical activity of the lateral rectus muscle was recorded simultaneously with the single unit activity from the striate cortex the sites of recording lying contralaterally to each other The phasic activity of the lateral rectus eye muscle corresponded in this case to the rapid phase of postcaloric horizontal nystagmus Occasionally the discharge rate of most visual units showed a change in relation to the nystagmic rhythm Only in few units was this relationship relatively regular during the whole period of postcaloric nystagmus Two main types of reaction could be found 1) Units that discharged in general correlation with the rapid phase of nystagmus 2) Units that often discharged fairly regularly during the slow phase of nystagmus but showed a decrease of the discharge rate during the rapid phase of nystagmus The results indicate that the visuovestibular integration can occur at least partly in visual cortex also in cases where the retinal input caused by light is excluded

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Vanha Perttula T P J (Department of Anatomy University of Turku Finland) ESTERASES OF THE RAT ADENOHYPOPHYSIS AS RELATED TO SECRETORY FUNCTIONS

Carboxylic esterases of the rat adenohypophysis were studied histochemically using thiocholine esters naphthol esters 5-bromoindoxyl acetate and thioacetic acid In their classification various modifier substances were used and they were also fractionated using centrifugation starch gel electrophoresis and DEAE-cellulose chromatography Quantitative determination of esterases was done in experimental conditions using four different substrates for separate enzymic activities Histochemical studies in fresh sections showed a fine granular E 600-sensitive activity in FSH LH and TSH-cells and a weaker one in STH-cells Short-chained (C—C) substrates were preferred The activities were markedly increased during augmented hormonal synthesis in the respective cells and decreased during the secretory quiescence In formalin fixed sections a pericytic E 600-sensitive activity preferred somewhat longer-chained (C—C) substrates It was increased during augmented secretory activity of FSH and LH cells and its contribution to the phagocytic functions of the pericytic cells is suggested The third E 600-sensitive activity in the epithelial cells between anterior and intermediate lobes showed increased activity after gonadectomy and adrenalectomy The significance of these cells is unknown

In formalin fixed sections a coarse-granular E 600-resistent activity was shown in FSH TSH STH and ACTH-cells with acetic acid esters only It was markedly increased in connection with the enhanced secretory activity of the respective cells and its contribution to the formation or release of the secretory granules is suggested

A weak nonspecific cholinesterase activity was shown in TSH-cells but its significance is unknown Capillary endothelial cells contained E 600-resistent esterolytic activities using thiocholine esters and acetylesters as substrates

Starch gel electrophoresis resulted in eleven different activities which could be correlated with the histochemical findings Quantitative studies showed significant changes during experimental conditions in correlation to the histochemical results

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Vapaatalo H I L. Ahtee and M K Paasonen (Department of Pharmacology University of Helsinki Finland) RELEASE OF CATECHOLAMINES BY SOME PHENOTHIAZINES FROM THE ADRENAL GLAND

It is known that chlorpromazine and related agents prevent the uptake of noradrenaline by granules of the adrenal medulla (Carlsson *et al* 1963) release 5HT from platelets and cause haemolysis of red cells (Ahtee and Paasonen 1965) In previous *in vitro* experiments (Ahtee and Paasonen 1965 unpublished) desmonomethylchlorpromazine relased more 5HT from platelets than chlorpromazine Chlorpromazine sulphoxide was also an active 5HT releaser but the quarternary *N hydroxyethylpromethazine* (NHP) was not

These phenothiazines were compared as releasers of adrenaline and noradrenaline from bovine adrenal medulla The gland was perfused with Thyrode solution at 37 C through an artery and 3 min samples of the *effluent were collected from a superficial wound The drugs were injected* close to the gland in a volume of 0.5 ml The amines were estimated spectrophotofluorometrically

Injection of 3×10^{-5} M to 3×10^{-3} M concentration of the solutions caused an increase in the release of both amines The release of noradrenaline was more persistent (up to 12 min) than that of adrenaline Chlorpromazine at 3×10^{-3} M was roughly equipotent with the same concentration of chlorpromazine sulphoxide but weaker than desmonomethylchlorpromazine at 3×10^{-4} M NHP at 3×10^{-3} M released adrenaline but hardly any noradrenaline

The presence of phenothiazines in the effluent was studied spectrophotometrically during 12 min perfusion after the injection The first 3-min samples contained more of phenothiazines than the following three 3-min samples together The amount of chlorpromazine and desmonomethyl chlorpromazine recovered was about 30 % that of chlorpromazine sulphoxide 60 % and that of NHP 70 % of the dose injected The amounts of phenothiazines retained in the adrenals were not related to the quantities of catecholamines released (Supported by USPHS Grant MH 5832)

Ahtee L and M K Paasonen *Ann Med exp Fenn* 1965 43 101—105
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Vartia A P Terho H Suomalainen T Peltonen and L Hirvonen (Cardiorespiratory Research Unit Department of Physiology and Department of Zoology University of Turku Finland) STRUCTURAL AND FUNCTIONAL DEVELOPMENT OF THE CHICK LUNG FROM THE SECOND WEEK OF INCUBATION UNTIL HATCHING

Chick embryos were dissected out from eggs at 7 to 20 days of incubation age Spontaneous hatching also occurred on the 20th or 21st day The lungs were fixed with Lillie s formalin alcoholacetic acid and paraffin sections stained by several methods the best results being obtained by Domagk s stain Observations were also made on the egg shell the air space the piping squeaking and respiratory movements of the embryo and the gross appearance of the lungs and their possible floating in the fixing fluid At the 10th day the lungs are divided into lobuli by connective tissue septa and each lobulus is formed around a parabronchus At the end of the 2nd week the boundaries of the lobuli become less distinct On the 20th or 21st day the lung tissue achieves a porous appearance due to the opening out of the vestibuli arising from the parabronchi and leading to the periphery of the lobuli The porous structure was found both before and after spontaneous hatching simultaneously with macroscopic signs of aeration of the lungs such as reddish colours rough (vesicular) surface and floating in fixative However porosity was not homogeneous throughout the lung and there was one hatched chicken with compact (nonporous) lungs Piping or squeaking does not presuppose porosity of the lungs but indicates air flow through the syrinx Intratracheal insufflation of air did not change the microscopic structure of the lungs at the 18th to 20th days Artificially hatched embryos were able to perform respiratory movements at a slow rate from the 20th day onward The respiration of the young embryo is effected through the allantois which takes up oxygen from the air entering through the porous egg shell Oxygenation of the pulmonary blood apparently starts gradually towards the end of the embryonic period Also air filling of the lungs usually precedes hatching

Viljanen A A (Institute of Physiology University of Helsinki Finland)

THE NEURAL CONTROL OF HUMAN RESPIRATORY MUSCLES

It has previously been shown that a distinct relationship exists between the electrical and mechanical activities in man s voluntary muscles (Lippold 1952 Bergstrom 1962) The same correlations have been demonstrated in the intercostal muscle of the rat (Bergstrom and Kerttula 1961) The preliminary experiments show that there exists a distinct relationship between the mechanical respiratory functions and the regulating electric activity in the human intercostal muscles (Viljanen *et al* 1964)

In further experiments the electrical activity of human intercostal muscles and diaphragm has been examined electromyographically during normal and carbon dioxide stimulated respiration This activity has been correlated to the mechanical functions of respiratory muscles

Bergstrom R M The relation between the integrated kinetic energy and the number of action potentials in the electromyogram during voluntary muscle contraction *Ann Acad Sci fenn* A 5 1962 93

Bergstrom R M and Y Kerttula On the neural control of breathing as studied by electromyography of the intercostal muscles of the rat *Ann Acad Sci fenn A 5* 1961.79

Lippold O C J The relation between integrated action potentials in a human muscle on its isometric tension. *J Physiol (Lond)* 1952.117 492

Viljanen A A H Poppius R M Bergstrom and M Hakumaki Electrical and mechanical activity in human respiratory muscles *Acta Neurol Scand* 1965 41 Suppl 13

Vuori I M. (Sport Research Unit University of Turku Finland) STUDIES ON THE PHYSIOLOGICAL EFFECTS OF A LONG SKI RACE IN YOUNG AND OLDER MEN

Long ski races and walks have become an important tool in counter acting the lack of physical effort of daily work The physical stress caused by the race is unusually high for the participants The aim of this investigation was to study some physiological and possibly pathological changes caused by a 94 km ski race in young and older men

The occupation social status and age were compared with the forms amount motivation and norms of physical activity The physiological studies were made before and after the race 26 subjects cycled by an ergometer and the pulse rate and ECG were recorded In most cases the exercise pulse rates were lower after the race at the same workload. In two cases a clearly pathological ECG was found at both times and in one of these the changes were more pronounced after the race Blood and urine samples were examined in 36 subjects Serum glutamic-oxalacetic transaminase and lactic acid dehydrogenase increased in most cases and the aldolase activity in seven cases after the race Circulating eosinophiles disappeared in all but one case Haemoglobin concentration decreased after the race in 21 cases remained the same in two cases and increased in two cases The changes in packed cell volume followed the same pattern The pH of urine was 8 after the race in one case in others 4—5 In seven cases acetone bodies in one case sugar in three occult blood in four erythrocytes and in seven cases free iron was found in urine after the race In no case was albumin found in urine In addition to these studies also blood lactate and the amount of haemolysis were determined

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Viljanen A A (Institute of Physiology University of Helsinki Finland)
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In further experiments the electrical activity of human intercostal muscles and diaphragm has been examined electromyographically during normal and carbon dioxide stimulated respiration This activity has been correlated to the mechanical functions of respiratory muscles

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Lippold O C J The relation between integrated action potentials in a human muscle on its isometric tension *J Physiol (Lond)* 1952 *117* 492

Viljanen A A H Poppius R M Bergstrom and M Hakumaki Electrical and mechanical activity in human respiratory muscles *Acta Neurol Scand* 1965 *41* Suppl 13

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Wahlstrom G (Department of Pharmacology University of Uppsala Sweden) ABSTINENCE SYMPTOMS AFTER ALCOHOL TREATMENTS IN THE RAT

The degree of excitation in the abstinence period after prolonged alcohol treatments has been measured with an anesthesia threshold method in male rats The threshold is defined as the dose of hexobarbital needed to obtain a burst suppression of 1 sec or more in the electroencephalogram The hexobarbital was infused with a constant rate of 0.25 mg/kg/ ec All changes are expressed as per cent of 3 pre-experimental thresholds

The alcohol was administered by two routes

1 Intraperitoneal injection of 1.5 g/kg in a 15 per cent (W/V) solution during the activity period The number of doses was gradually increased up to three times daily

2 Oral administration of alcohol mixed in the drinking waters Two administration schedules were used Continuous access to a 20 per cent solution and intermittent daily access during the activity period to 10 or 15 per cent solutions for only two hours No other drinking water was given

A gradual increase in threshold which had reached 450 per cent (S E =67 N=4) on the fifth abstinent day was seen after the most prolonged (4 weeks) i p experiment A shorter treatment (2 weeks) gave a maximum of only 116 per cent (S E =54 N=11) on the same day Intermittent oral administration for two and six weeks also gave an increased threshold with a maximum on the fifth and fourth abstinent day The maximum increase was 219 (S E =5.2 N=6) and 264 (S E =67 N=7) per cent respectively In the longer experiment the maximum was flatter than in the shorter experiment Continuous access to alcohol for corresponding times gave lower increases in thresholds less abstinence excitation although the total consumption of alcohol was larger

An increase in a hexobarbital threshold has thus been found after various alcohol treatments in the rat This increase seems to have a maximum around the fifth day without alcohol

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Wahlstrom G (Department of Pharmacology University of Uppsala Sweden) RESETTING EFFECT OF ENFORCED LIGHT ON THE SELFSELECTED CIRCADIAN RHYTHM IN THE CANARY

Male canaries kept singly have been used to record the selfselected circadian rhythm Inside the cage there is a perch which regulates the light When the bird hops onto the perch the light goes out and when it leaves it the light is turned on The canaries can thus choose between light and darkness There is usually only one activity period (light) and one rest period (darkness) in the circadian period The circadian period is counted from the start of the activity (waking up) (Wahlstrom 1964)

In nine experiments the light was turned on by the experimenter 1 hour before the expected waking up Thus enforced waking up did not affect the duration of the following circadian periods Activity and rest were also uninfluenced The onset of light was thus taken as a starting point for the new circadian period the rhythm was reset by one hour Enforced light for 2 hours gave a reset of approximately 2 hours (N=9) Enforced light for 4 hours gave a reset of the circadian rhythm which was only about 2 hours (N=13) Thus the onset of light in this case was not taken as a new starting point

Remarkably similar results in the opposite direction have been obtained with enforced periods of darkness covering the first part of the intended activity (Wahlstrom 1965) At the time of waking up it is thus possible to cause on immediate rest of the circardian rhythm If the change in either direction in onset of light is 1—2 hours an equally large reset is immediately obtained

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Wallentin I (Department of Physiology University of Goteborg Sweden)

THE IMPORTANCE OF TISSUE PRESSURE IN SMALL INTESTINE AS A COUNTERFORCE AGAINST EDEMA FORMATION

The small intestine has about a 10 times larger capillary surface area than the skeletal muscle as deduced from determinations of the capillary filtration coefficient (CFC) This big capacity for transcapillary fluid transfer must be a prerequisite for the specialized functions of the mucosa as regards secretion and absorption It implies however the potential danger of creating severe intestinal edema whenever capillary pressure is raised e g by increased venous resistance The effect of prolonged elevations of venous outflow pressure was therefore studied on cats However the small intestine is evidently normally well protected against edema because during elevation of venous pressure the outward filtration rate decreased fairly rapidly with time and a new isovolumetric state was often reached These events point to a shift of the Starling equilibrium and in order to analyse the factors involved an indirect method was used consisting of stepwise raising and lowering the venous pressure During this procedure a given venous pressure could produce very different rates of net fluid transport depending on whether the pressure was reached from a lower or from a higher venous outflow pressure In most of these cases even the direction of fluid transport was reversed from filtration to absorption The transcapillary forces responsible for these changes of direction and extent of net fluid transfer could reach figures up to 14—15 mm Hg It could be deduced from available data that dilution of interstitial proteins could only be a minor part of these transcapillary forces and that increasing tissue pressure is the most important counterforce for the protection of the small intestine against edema

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Zade-Oppen A M M (Institute of Physiology University of Uppsala Sweden) ON POSTHYPERTONIC HAEMOLYSIS

Posthypertonic haemolysis is here defined as the haemoglobin liberation that occurs after red cells have been transferred from a hypertonic to a less hypertonic (isotonic) medium

It was found that posthypertonic haemolysis was proportional to the degree and duration of hypertonicity

Lovelock s (1953 a) statement that concentrations under 0 8 M NaCl do not cause posthypertonic haemolysis was confirmed

The results were not the same with different electrolytes

Posthypertonic haemolysis was also obtained after suspension in 1 4 m sucrose in a medium with isotonic electrolyte concentration this haemolysis being less than with hypertonic electrolytes

A possible explanation of the differences found is that electrolytes permeate partially into the cells the amount penetrating depending upon the particular electrolyte while sucrose may penetrate to a smaller extent or not at all The partial permeation of NaCl was experimentally verified Due both to the shrinkage of the cells in the hypertonic medium and to the entrance of some solute there will be a large concentration gradient between cell interior and medium when the cells are returned to an isotonic medium The size of this gradient may be the decisive factor for the degree of haemolysis

In contrast to the findings of Lovelock (1953 b) glycerol inhibited posthypertonic haemolysis This means that anti haemolytic action of glycerol when blood is preserved by freezin may be due to two reasons Not only does glycerol decrease the mechanism originating posthypertonic haemolysis i e the increase of tonicity due to separation of pure ice crystals but it may also inhibit the haemolysis as such

Inhibition was also obtained with dextran and raffinose These effects suggest that posthypertonic haemolysis may be colloid-osmotic in type (Willbrandt 1941) i e inhibition is obtained by balancing the osmotic pressure of internal haemoglobin with an extracellular nonepenetrant solute

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Zimmermann-Nielsen, C. (Department of Biochemistry University of Aarhus Denmark) **OBSERVATIONS ON THE PATTERN OF AMINO ACIDS IN THE INTESTINAL CONTENTS AFTER INTRODUCING PROTEINS IN THE SMALL INTESTINE OF CATS**

In 1957 Nasset put forward the hypothesis that the absorption of dietary proteins from the small intestine takes place from an amino acid mixture (an "amino acid pool") in which the mutual ratio between the amino acids is constant and independent of the amino acid composition of the dietary protein. Since 1957 Nasset and co-workers (Nasset and Ju 1961 Nasset 1962 Ganapathy and Nasset 1962) have carried out investigations on dogs and rats and always obtained results which seem to support the proposed hypothesis.

In connection with studies on the absorption of proteins from the small intestine of cats we had the opportunity to investigate the free amino acids in the intestinal contents. The observations include studies on the following proteins gelatine casein and edestin. The free amino acids in the intestinal contents were determined quantitatively 60 minutes after introducing the protein in the small intestine of anaesthetized cats. Marked differences between the relative ratios of amino acids in the intestinal contents (expressed as the percentage ratios of the examined amino acids) in the various series of experiments were found and as the agreement between duplicate experiments proved to be rather good it is justifiable to draw the conclusion that the "amino acid pool" in the small intestine of cats is of very different composition after introducing each of the proteins mentioned. The pattern of free amino acids in the intestinal contents obtained from various experiments is compared to the amino acid pattern in hydrolysates of the proteins under investigation. With gelatine essential similarities are found between gelatin s amino acid composition and that of the intestinal contents after introduction of the same protein.

It appears from the results that under the applied experimental conditions and with cats as the animal used for experiments no support for the theory of Nasset is found.

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Aberg H (Institute of Physiology and Medical Biophysics University of Uppsala Sweden) **CAPILLARY PERMEABILITY**

Investigations of the permeability of capillary walls have followed two general lines The separation of lymph from plasma has been considered as due to sieving in the pores of the capillary membrane (Pappenheimer 1953 Renkin 1954 Grotte 1956) It has also been suggested that the membrane is a flowing i e continually changing structure The cell walls invaginate and form first small lacunae which later become isolated vacuoles in which matter is transported to the intercellular space (Bennett 1956)

Winne (1956) considers that a combination of sieving and vacuole transport is the most probable molecular transport mechanism Assuming that (Starling s law) the intra-capillary pressure is higher on the arterial side and lower on the venous side than the tissue pressure these gradients will determine the direction of the hydrodynamic flows A flow of lymph means that the outflow from the arterial side of the capillary exceeds the inflow on the venous side A solute entering the arterial side of the system escapes from the capillary partly by diffusion and partly in a bulk flow On the venous side the molecular properties of the solute by size determine its behaviour A small solute with a high diffusion coefficient may be able to diffuse out of the capillary against the entering hydrodynamic flow which will stop the escape of larger species but for similar reasons a large species lying outside the capillary will have a greater chance of moving into the capillary against a concentration gradient (Herz diffusion) Those phenomena have been demonstrated across single membranes (Garby 1957 Rapoport 1965) They have also been shown in a simple model system with three cells separated by two membranes It is further possible that this mechanism is operative in animal tissues and contributes to molecular separations across the capillary walls

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Ablad B M Brogård B Duce L Ek L Garberg and B Johansson (AB Hassle Molndal Sweden) **PHARMACOLOGICAL PROPERTIES OF A NEW BETA ADRENERGIC RECEPTOR ANTAGONIST H 56/28**

The compound H 56/28 (1 (o-allylfenoxi) 3-isopropylamino-2 propanol hydroklorid) was found to be an active beta adrenergic receptor antagonist in studies on isolated papillary muscle and on the cardiovascular system of cat and dog H 56/28 had about the same beta receptor blocking activity as propranolol and was approximately 10 times more potent than pronethalol H 56/28 and pronethalol were found to posses weak intrinsic beta adrenergic stimulating properties whereas propranolol was devoid of such an action In relation to the beta blocking activity the intrinsic heart stimulatory effect of H 56/28 was weaker than that of pronethalol High doses of H 56/28 propranolol and pronethalol elicited a direct cardiac depression not related to beta receptor blockade

Studies of the beta receptor blocking effect of the two optical isomers of H 56/28 indicated that the laevo isomer was twice as active as the racemate whereas the dextro isomer was about 25 times less active The direct cardiac depressant effect of the two isomers was approximately equal

Strophantine induced ventricular tachycardia in unanaesthetized dogs was reverted by both isomers of H 56/28 the dextro isomer being some what more potent

Ahman, T and F Makela (X ray and Laboratory Department Laaketukku OY Helsinki and Cardiorespiratory Research Unit Department of Physiology University of Turku Finland) IMPROVED METHOD OF PAPER PRINTING OF ECG RECORDED ON TAPE BY HOLTER—AVIONICS SYSTEM

The most significant source of error in the reproduction of ECG recorded on tape by the Holter—Avionics system is the distortion of the signal due to a short time constant The tape recorded ECG is correctly displayed by photographing the complexes from the monitor of an Electrocardioscanner (Avionics Research Products TM) i e tracing resembles the ECG recorded simultaneously and the rectangular calibration signals are rectangular Avionics Corporation produces an analysing equipment (Electrocardiocharter TM) with which the tape recorded ECG signals can be displayed on paper The limitation of this apparatus is the shortness of the time constant The tape recorded ECG signals from the output sockets of Electrocardioscanner TM may be recorded correctly using normal DC amplifiers (e g Mingograph 24) provided that an additional circuit is used before the DC-amplifier This additional circuit is constructed for each separate case and is thus not a convenient method for continuous use To avoid the difficulties described an amplifier-demodulator unit (MV I) was constructed The principle of this apparatus is as follows In the first phase the ECG signal obtained from the output of Electrocardioscanner TM modulates the carrier wave with regard to frequency and the frequency modulated signal produced is recorded with a high fidelity tape recorder The speed of both tapes is 19 cm per sec In the second phase the speed of the tape with the recorded signal is reduced to 2 475 cm per sec and the ECG signal is demodulated in MV I unit A paper tracing is recorded with a DC-amplifier and a direct writer A rectangular calibration signal is displayed correctly by this equipment Samples of ECG tracings obtained by different methods are demonstrated

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Astrand I (Institute of Work Physiology Stockholm Sweden) EVALUATION OF THE CIRCULATORY LOAD DURING HOUSEHOLD WORK.

The circulatory load was determined on 10 healthy housewifes 20—45 years old both at submaximal and maximal work levels on a bicycle ergometer The heart rate was followed continously when performing the work in the home Predicted from these data they used 42 per cent in mean of their aerobic work capacity during the occupational job This percentage figure is of the same size as for other occupational jobs in which one s own body has to be moved These results indicate that one individual automatically chooses a certain level regarding the physical strain during work of this type

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Astrand P-O, [redacted] Ekblom B Gullbring H Kirschner and M Rapport (Department of Physiology Gymnastiska Centralinstitutet Stockholm Sweden) HAEMODYNAMICS DURING SUBMAXIMAL AND MAXIMAL WORK AFTER BLOOD LOSS AND REINFUSION OF RED CELLS

In five subjects several work loads were performed up to maximum at sea level and 4 000 m simulated altitude (P_{Bar} 462 mm Hg) (i) under normal conditions (ii) two days after a blood loss of about 500 ml (iii) 3 weeks after reinfusion of the red cells which had been stored Cardiac output (dye dilution technique) oxygen uptake heart rate pulmonary ventilation blood lactates intra arterial blood pressure arterial oxygen saturation and capacity were determined Before the various experiments the red cell mass (Cr^{51}) and plasma volume (J^{125}) were measured The results will be discussed

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Astrom A and J Crafoord (Department of Physiology Karolinska Institutet Stockholm Sweden) AFFERENT IMPULSES IN KIDNEY NERVES

Afferent nerve impulses have been recorded in the kidney nerves of cats and rats Under average experimental conditions the afferent activity was low or absent but its frequency could be greatly increased e g by elevation of renal vein pressure The response to venous stasis was enhanced during intravenous infusion of mannitol or Ringer s solution The activity seemed to originate from slowly adapting receptors It is suggested that the receptors are activited by increased tenseness of the kidney The physiological significance of this afferent inflow will be discussed

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ANALYSIS OF SPONTANEOUS SPIKE POTENTIAL ACTIVITY IN DEVELOPING RABBIT DIENCEPHALON

BY

JUHANI HYVARINEN

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JUHANI HYVARINEN

HELSINKI 1966

PREFACE

This study was carried out during the years 1963—66 at the Institute of Physiology, University of Helsinki.

I wish to thank my teacher, Professor R. M. Bergstrom, M. D., Head of the Institute. It was at his suggestion that I embarked upon this analysis of spontaneous unit activity in the developing diencephalon. He introduced me to the problem and followed my work with great interest, placing the research facilities of the Institute at my disposition. I also wish to thank him for his valuable criticism of the investigation during its different phases.

My thanks are due to Mr Kjell B. Doving, Ph. L., Department of Physiology, Karolinska Institutet, Stockholm, for advice on the microelectrode technique. I am also grateful to Mr Roger Blåfield, M. Sc., and Mr Erkki Kurenniemi, for advice concerning electronic equipment, and Mr Aarne Halme, for planning the digital system for impulse interval measurement. The interested assistance given by Mr Timo Heino, B. M., Mr Jussi Haikara, B. M., Mr Antti Poranen and Mr Anssi Sovijarvi has been of great help in the making of this investigation. The assistance of Mrs Marjut Österberg is gratefully acknowledged. May I also extend my thanks to the whole staff of the Institute of Physiology for their help in various parts of this work.

I am extremely indebted to Mr Juha Partanen, L. Pol. Sc., and Mr Anders Ekholm, M. Pol. Sc., for their introducing me to the field of time series analysis and their constant help in the statistical analyses of this work. They developed the procedures for the statistical computations in this study. I also wish to thank Mr Kari Kärkkäinen for writing the computer program for these analyses. The computations were effected in the Computing Centres of the Institute of Technology, Helsinki, the Institute of Nuclear Physics at the University of Helsinki and the Finnish State Computer Centre. I wish to thank these computing centres for pleasant cooperation, and plenty of computer time.

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JUHANI HYVARINEN

CONTENTS

INTRODUCTION

The role played by the spontaneous activity of the receptors and the brain has been a subject of discussion since HELMHOLTZ (1867) introduced the concept of visual "Eigenlicht", which described the light dust of a dark visual field. In GRANIT's (1955) review of this spontaneous activity he concludes that it is important as regards the general excitability of the nervous centres and also for the sensory discrimination system as a carrier, being modulated by presented stimuli. On the basis of experimental evidence derived from sensory physiological experiments with human beings as subjects, BERGSTRÖM (1964) demonstrated the possibility of defining subliminal sensory quantities, which he related to the spontaneous activity of the corresponding sensory system, as imaginary quantities. According to him, the suprathreshold sensory manifold (REENPÄÄ 1962) can be defined with the aid of real numbers. In many recent writings, the spontaneous activity has been termed "neural noise" (e.g. VERVEEN and DERKSEN 1965, see also GERARD and DUYFF 1962). Many workers have found the variability of neuronal impulse sequences to be random in nature and the impulse sequences have shown characteristics of a Poisson process at low stimulus intensities and in a spontaneous state (BULLER, NICHOLLS and STRÖM 1953, HAGIWARA 1954, GROSSMAN and VIERNSTEIN 1961, BISCOE and TAYLOR 1963). In some recent reports, divergences from a Poisson process in spontaneous and driven activity have been found by WERNER and MOUNTCASTLE (1963) and POGGIO and VIERNSTEIN (1964). In their studies, the neuronal activity displayed dependence between the impulse intervals.

Currently, the meaning of the different types of impulse sequences is largely unknown. In all complex physiological systems there occur great variations which are difficult to relate to the different potential functions of these systems. During the maturation of the brain, this complexity of the system can, however, be supposed to change and various functional stages are observable (BERGSTRÖM 1963 a, b). Thus, in this study,

the neuronal spike activity was investigated in different maturational stages of the experimental animal. If many different functional stages are to be found, the study needs to be started at an early phase of development. The recording of single-cell activity in intra-uterine conditions presents considerable difficulties; consequently a study of the postnatal development was considered most suitable, the rabbit being chosen as an experimental animal by virtue of its being born in a rather immature state. Examination was focused on the thalamic non specific and other nuclei, and neighbouring regions were studied for comparative purposes. The thalamus was chosen, as it is an important relay station in sensory information processing in the brain. The role of the spontaneous activity of the thalamus might be of importance in respect of both general excitability and sensory discrimination.

The neuronal impulse sequences have mostly been studied in terms of the interval distributions. This was done in newborn rabbit brain in a preliminary investigation by BERGSTRÖM, HYVÄRINEN and KURENNIEMI (1966). The histograms observed faintly resembled gamma distribution although they differed significantly from it.

The interval distributions give information based on interval lengths alone. Additionally, information about the sequential order of intervals was sought in this investigation by computing autocorrelations and intensity functions to investigate the dependences between intervals.

Methods which allow of recording without anaesthesia are difficult to apply to newborn animals. Anaesthesia was used, but so regulated that differences between age groups should not arise as a consequence. It is known that anaesthesia induces changes in impulse sequences (MOUNTCASTLE, DAVIES and BERMAN 1957, WERNER and MOUNTCASTLE 1963) which must be borne in mind on interpretation of the findings in this study.

LITERATURE SURVEY

During the past fifteen years a gradually growing interest has been noticeable in the analysis of variability in sequences of neuronal events, although the literature on this subject is still rather sparse. This interest has been methodically related to the development of microelectrode recording and digital analysing techniques.

FATT and KATZ (1952) demonstrated the presence of a spontaneous subthreshold activity of random nature in the motor nerve endings. They showed that possibly in the smallest endings, thermal noise could be the source of this activity. BULLER, NICHOLLS and STRÖM (1953) found that on the application of different stimulating tensions to frog muscle spindle the impulse intervals were at very low discharge rates exponentially distributed and thus formed a random sequence. The dispersion of intervals was less at higher discharge rates. The same thing was proved independently on the muscle spindle of Japanese toad by HAGIWARA (1954). KUFFLER, FITZHUGH and BARLOW (1957) examined the spontaneous activity of the cat's retinal ganglion cells, and found that the impulse interval distribution was a gamma distribution. HUNT and KUNO (1959) examined spinal interneurons intracellularly, and observed that the short impulse intervals happened at random but that a certain rhythm prevailed at longer intervals. A similarly timed fluctuation of the membrane potential was discernible. BISCOE and TAYLOR (1963) examined the discharge patterns in afferent fibres from the cat carotid body, with normal circulation, and during perfusion with saline. The distribution of intervals was exponential in both the normal and the abnormally stimulated state. The impulse intervals thus showed characteristics of a Poisson process. GROSSMAN and VIERNSTEIN (1961) studied the spontaneous and acoustically stimulated activity of cells in the cat's cochlear nucleus. The interval distribution was exponential in 30 of the 31 cells studied without regard to the stimulus intensity. RODIECK, KIANG and GERSTEIN (1962) found in the cochlear nucleus of the cat exponential, nearly Gaussian,

bimodal, scaling invariant and unclassified distributions GERSTEIN and MANDELBROT (1964) developed a random walk model based on sub threshold "random walk" of the membrane potential between depolarization and hyperpolarization With this model, they could simulate the impulse series recorded by RODIECK, KIANG and GERSTEIN (1962)

WERNER and MOUNTCASTLE (1963) examined the variability of spontaneous and driven activity of thalamic somatic sensory neurons in unanaesthetized monkeys and remarked that the impulse interval sequences were very seldom stationary in the strict statistical sense, when short succeeding sequences were investigated with the analysis of variance The lack of stationarity was mainly related to cyclic changes in the impulse sequences, revealed by analysis of the autocorrelation function

POGGIO and VIERNSTEIN (1964) extended this analysis by computation of the expectation density of impulse sequences They found periodic oscillations in the expectation density of spontaneous and driven neuronal activity The frequency of these oscillations varied jointly with the stimulus intensity In most cases, the periodic character of the expectation density was dependent on the sequential order of the impulse intervals, and was lost if the intervals were shuffled into a stochastic order

HERZ CREUTZFELDT and FUSTER (1964) examined the spontaneous activity in the ascending visual system, and described the activity in the optic tract as being faster, and interval distributions as tending towards exponential ones in the ganglion geniculatum laterale the activity was slightly slower the distributions being multimodal, and in the visual cortex the spontaneous activity was even slower, and the form of the histogram intermediate HEISS and BORNSCHEIN (1965) examined the spontaneous activity of fibres in the optic tract, and the influence of diffuse light ischaemia, strychnin and barbiturate on this activity They noticed that ischaemia accelerates the activity of an optic fibre and makes it more regular Diffuse light on the whole retina slows down the activity, and makes it irregular intravenously injected strychnin accelerates it and pentothal retards it

VERVEEN and DERKSEN (1965) studied the fluctuations of the membrane potential in different frequency bands The amplitude of the fluctuations followed the 1/f law and these were thus not attributable to thermal noise They also suggest a possible explanation of different forms of post stimulus latency histograms based on the fluctuation of the membrane potential about the threshold value According to this the greater the stimulus intensity and the depolarization level, and the smaller

conversely, the smaller the depolarization level (the nearer the threshold), the more skewed is the distribution approaching exponential form

SMITH and SMITH (1965) analysed the spontaneous activity of the cortical cells in unanaesthetized cat isolated forebrain, and described the impulse interval sequences as a mixed process of two Poissonian processes STEIN (1965) showed by computer simulation that with random subthreshold input to a neural cell the output could in most cases be a gamma distribution if suitable parameters were selected BISCOE and TAYLOR (1965) showed that the refractory period may lengthen the shorter intervals with fast frequencies, and thus bring about a gradual ascending phase of the distribution

As in this study changes in the impulse sequences are sought during the maturation of the brain, a brief survey of the literature of development of the electrical activities of the brain is presented below Special attention is paid to findings related to the postnatal development of the electrical activities of the receptors and the brain in the rabbit, chosen as the experimental animal

HUNT and GOLDRING (1951) showed that the response of the visual cortex to electrical stimulation of the optic nerve in rabbits is evident at birth The cortical light response appeared on the 7th postnatal day A progressive change in the form of the response occurred between the 1st and 21st days from birth ANGGARD (1965) demonstrated the presence of cochlear receptor potentials in the rabbit on the 5th postnatal day These potentials gradually changed towards the adult type until the age of 15 days when they attained a steady level

BRADLEY et al (1960) observed that electrical activity was first present in the rabbit cerebral cortex on the last (= 31st) day of gestation On the 7th postnatal day, some interhemispheric synchrony was noted GARMA and VERLEY (1965) conclude that in the rabbit, cortical generators are active on the second week of extrauterine life DO CARMO (1960) obtained classical recruiting response after 15 days of age in the postnatal rabbit when the nucleus ventralis anterior thalami was stimulated At 18 days, the direct cortical response was approximately of adult duration

The presence of an arousal reaction in the electrocorticogram has been shown by BERNHARD KAISER and KOLMODIN (1959) in the foetal sheep at the time of birth and by BERGSTROM (1962) in the foetal guinea pig at the same time As these animals are born in a well matured state in comparison with the rabbit which is very immature at birth, the time of birth of sheep and guinea pig would correspond in the rabbit to the age of one to two weeks

SCHADE (1960) observed that the unit activity in the rabbit cerebral

cortex appeared between the 5th and 10th days of age PURPURA, SHOFER and SCARFF (1965) reported long lasting action potentials, and synaptic potentials in newborn kitten neocortex The intracellularly recorded action potentials lasted up to 10 msec NAKA (1964), in his studies of foetal kitten spinal cord, recorded extracellular spikes of great amplitude, up to 30mV and more, and considered them as "giant extracellular responses' of FREYGANG and FRANK (1959), which could be capacitatively coupled to the recording electrode across an inactive point of the cell membrane

METHODS

Material

According to the publications cited above many of the electrical activities of the brain develop postnatally in the rabbit Young rabbits are greater in size than mice and rats and thus the difficulties which arise in stereotaxic methods might be assumed to be less in the rabbit. Young rabbits are available throughout the year which makes them more suitable than kittens The rabbit was thus chosen as the experimental animal Rabbits of both sexes were used.

The successful recording of single-cell activity was effected in 64 rabbits (of a total of 89 in respect of which recording was attempted) ranging in age from one hour to eight months i e from newborns to young adults (SANDFORD WILSON and MUIR 1957) They ranged in weight from 35 to 3 440 g Their ages are given in Table 1 The birth of all animals less than three months old at the time of recording took place between the 30th and 33rd days of gestation

Anaesthesia

The recordings were made under urethan anaesthesia with thorough local anaesthesia of the places of wounding and the pressure points Urethan was used because it is well tolerated by both young and adult rabbits in acute experiments It was administered in the form of 20 per cent water-solution Half of the dose was given intramuscularly and the other half intraperitoneally a suitable rate of induction of anaesthesia was thus achieved. An attempt was made to achieve a suitable level of anaesthesia by observing the state of the animal the breathing rate the electrocorticogram, electrocardiogram and the withdrawal reflex. The dosage of the anaesthetic was so regulated that the ECoG showed patterns of sleep and that a slight withdrawal reflex was present on the animal being nipped hard with forceps The mean doses in different age groups to produce similar conditions of anaesthesia are presented in Table 1 The initial dose producing similar conditions of anaesthesia was slightly greater in the age group 69—84 days. Smaller additions of 10—20 per cent of the initial dose were given intramuscularly during experiments The animals would accordingly remain quietly sleeping during the whole experiment which took 5—10 hours

The electroencephalographic signs of urethan anaesthesia (LONGO 1962 p 111) were found to be rather reliable in rabbits more than one month old. In younger rabbits the basic configuration of the ECoG undulates very slowly (BRADLEY et al 1960) and is not so clearly changed by anaesthetic agents In rabbits more than two weeks old slow waves of great amplitude were recorded under the urethan anaesthesia these changed to quicker waves of smaller amplitude as the effect of urethan passed away In rabbits less than two weeks

Fig. 2 Histological localization of the recording site. A frontal section of the brain of a 10 day-old rabbit. To the left electrode track is seen to traverse the right side cortex, hippocampus and thalamus. To the right is shown the location of the electrode tip at the end of the experiment in another section from the same brain with greater magnification (21.5 ×). The four marking holes referred to in the text are seen at the margins. The nearly vertical and horizontal lines joining the opposite marks cross each other at the site of the electrode tip, in this case in the right pedunculus cerebri

In the identification of different structures use was made of the atlases of FIFKOVA and MARŠALA (1962), SAWYER, EVERETT and GREEN (1954) and WINKLER and POTTER (1911). Table 2 presents the regions in which at least 9 statistically analysed cells were located. The abbreviations to be used for the regions are also given.

TABLE 2

Localization of statistically examined cells

Regions / Age days	Hp	Am	CP	CI	ZI	R	NCL	VA	VL	LA	LP	Total
0–13		8	1	1	2	2	—	—	—	—	—	14
14–33	8	9	6	23	6	29	2	10	7	4	5	109
49–84	1	—	9	10	—	14	—	—	—	1	—	35
157–255	1		—	4	9	15	7	16	19	11	4	86
Total	10	17	16	38	17	60	9	26	26	16	9	244

Additionally 15 cells were histologically localized in n. caudatus putamen, n. anteroventralis thalami, n. geniculatum laterale, area hypothalamica lateralis and lemniscus medialis. 39 cells were not histologically localized, 36 of them in age group 0–13 days. Abbreviations: Hp = hippocampus, Am = amygdala, GI = globus pallidus, CI = capsula interna, ZI = zona incerta, R = n. reticularis thalami, NCL = n. centralis lateralis, VA = n. ventralis anterior, VL = n. ventralis lateralis, LA = n. lateralis anterior, LP = n. lateralis posterior

Data reduction and selection of samples

For visualization of the pulse intervals in a concise and persisting form the following method of photography was developed (Fig 1 b) A capacitor was charged from a 1.5 V battery through a variable resistor The nerve impulses triggered a Tectronix 161 pulse generator of which the output was employed to drive a relay which short circuited the capacitor with a slight delay when a pulse occurred The same pulse triggered another pulse generator with a shorter delay of which the output was employed to modulate the z axis intensity of the oscilloscope An unblanking spot was thus formed at the end of each impulse interval on the corresponding voltage level The height of this spot from the baseline is a measure of the length of the preceding interval The time constant could be varied by means of the resistor The non linearly increasing voltage allowed of the detection of changes in both short and long intervals (Fig 7) All the recorded material was photographed in this way on a slowly moving film Calibrations were taken from a Tectronix 162 pulse generator

These photographs were of great help in interpreting the results of the time series analyses The neural interval sequences are seldom stationary when tested by ordinary statistical methods (WERNER and MOUNTCASTLE 1963) These photographs were used to eliminate impulse sequences with an obvious lack of stationarity i e those exhibiting changes in the mean discharge rate or in dispersion of the interval lengths The vertical bars in Fig 7 indicate where such changes were observed As a stationary sample there was accepted the sequence of intervals between these bars The longest stationary sample from every cell was selected for statistical analysis The beginning points and the durations of these samples were measured from the film and they were localized on magnetic tape with a stopwatch

The measurement and digitization of the interval lengths were effected with an ordinary general purpose computer (Elliott 803) as follows (Fig 1 c) The impulses in the stationary sequences fed from the tape recorder were monitored in the loudspeaker and on the oscilloscope and fed through the 2 A 60 amplifier to trigger a 161 pulse generator The output of the pulse generator was fed into a counter and into a flip-flop The output of the flip-flop was fed into the number generator of the computer which had been suitably prepared The flip-flop output voltage level determined the sign of the contents of the number generator The computer was programmed to check the sign of the number generator and add one between the checks to a memory address When the sign was reversed following the occurrence of an impulse the address was changed Thus the lengths of the intervals were stored in the computer memory The time measuring cycle consisted of three machine loops each 720 microseconds in length The discretization interval was thus 2 16 milliseconds Sequences in which intervals of less than 5 milliseconds were observed were fed into the computer at half speed from the tape recorder Shorter intervals than 1 5 msec were not found in this material In the event of very slow activity the speed of the tape recorder was increased to save computer time The intervals thus changed were multiplied by the corresponding factors $^1/_2$ 2 4 or 8 later in the analysing program

The output of the digitized intervals was on punched paper tape from the computer memory The coding numbers of the cells were printed on these tapes prior to every sample these consist of five digits The two first give the number of the animal in the experimental series the third the number of the electrode track in one experiment and the last two the number of cells along that electrode track These paper tapes were then analysed with a more rapid computer (Elliott 503)

Recordings were made of the spontaneous activity of about 500 cells Of these 298 showed a stationary function with a sufficient number of impulses and a high signal-to-noise and artefact ratio which would permit of statistical analysis of the interval sequences Altogether

411 579 impulse intervals were analysed In the age groups of less than two weeks difficulties were encountered as a consequence of the very slow mean discharge rate This would call for a long recording time to ensure an adequate number of impulses for statistical analysis During such periods of 20—30 minutes it is very likely that there occurs a change in the function of the cell Accordingly in the lowest age groups the stationary series often contain few intervals (minimum 66) Usually more than 1 000 intervals were analysed per cell (mean 1 400)

All the 298 statistically analysed samples were used in mutual comparisons of the age groups In the studies of different brain regions the histologically localized 244 cells presented in Table 2 were employed For a further 53 cells located in the same general region which did not provide adequate samples for the statistical analysis but where the recordings were free from artefacts measurement of the mean impulse frequency was made with the counter and stopwatch. Thus the cells in which the mean impulse frequency was measured numbered 351 in total The cells that were not histologically localized were situated in the same brain regions on the basis of their stereotaxic coordinates although the individual nuclei of these cells are not known with certainty

RESULTS

GENERAL MEASUREMENTS

Distance between spontaneously active cells

In the young rabbits it was often difficult to find spontaneously active cells In 8 of the 24 rabbits less than 6 days old no spontaneous activity was found in spite of several electrode penetrations through the brain regions studied Activity provoked by the advancing electrode was present in these cases

The distance from a recorded cell to the nearest other spontaneously active cell along the same electrode track was measured with the manipulator to a degree of accuracy of ten microns If only one cell was recorded for each penetration, the distance from this cell to be cortical surface was taken to be the measurement for that cell In such cases, this distance was long as compared with the distances between active cells The means and standard deviations of these distances are given in Table 3 This mean distance diminishes slightly with increasing age The dispersions are rather large, which indicates that there is a great deal of variation in the probability that an active cell happens to be just on the track of the electrode tip The spikes are generally held as being recorded from the pericarya (VALLEALA 1961) As the distances between pericarya become greater with increasing age (SCHADE 1960), the diminution in distance between active cells indicates that more cells become active with increasing age

Extracellular action potentials

In the whole material, initially negative spikes were of less frequent occurrence than initially positive ones In rabbits less than two weeks old, initially negative spikes were very rare none being found in rabbits

TABLE 3

Mean distance from a cell to the nearest other recorded spontaneously active cell along the same electrode track in different age groups

AGE days	NUMBER OF CELLS	MEAN DISTANCE mm	STANDARD DEVIATION mm
0–6	20	1 50	2 26
7–22	94	1 14	1 68
26–84	95	1 33	2 06
157–207	32	0 68	0 81
212–255	57	0 75	1 31
Total	298	1 10	1 78

less than 7 days old The amplitudes of the negative spikes were usually less than a millivolt, but sometimes ranged to 1 5 millivolts The initially positive spikes were usually greater in amplitude, ranging from 200 microvolts to 3 millivolts, and occasionally up to 10 millivolts peak to peak Even in such cases, the spikes were clearly biphasic, and could thus be considered extracellular They could possibly be interpreted as the 'giant extracellular responses' of FREYGANG and FRANK (1959) observed by NAKA (1964) in foetal spinal cord Such spikes were mostly encountered in young animals less than two weeks of age

The duration of the extracellular action potential was measured visually fro the oscilloscope screen from the beginning of the first to the end of the se ond phase (Fig 3) In the higher age groups, the duration of the spike was in the normally reported range for adult mammals, msec In the age groups of less than two weeks, a markedly uration of the spike was often encountered However, in the same another spike might be of normal duration In this study, the duration of the recorded spike was 10 milliseconds Such long were often also of great amplitude The so called giant extra lar responses were often of long duration When on occasion, a cell biting such long action potentials was penetrated without noticeable urs to the cell, the intracellularly recorded action potential was of he same duration According to LORENTE DE NO (1947), the extra cellular initially positive biphasic spike should represent the derivative of the intracellular action potential Accordingly no major discrepancy

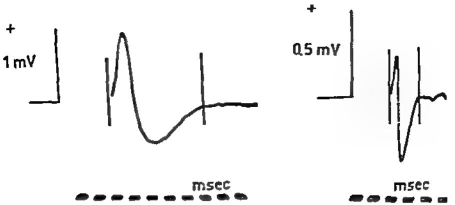


Fig 3 Configurations of extracellular action potentials To the left an initially positive action potential from a 9-day-old rabbit The duration was measured between the vertical bars and was in this case 5 5 msec To the right an initially positive action potential from a 2 month-old rabbit with a duration of 2 0 msec

between the durations of intracellular monophasic potential and extra cellularly recorded initially positive biphasic potential should be expected, when the distance of the electrode from the recorded neuron is not very long

The means of the durations of the extracellularly recorded action potentials were calculated for different age groups and are shown in Fig 4 It is seen that the mean duration of the action potential is large until the age of two weeks, and then quickly diminishes to the adult values When individual values were examined, the greatest change was seen to occur on the 14th day of age On that day, the individual values of the duration clearly became less than in lower age groups The dispersion of the duration is great in low age groups which can well be explained, as in the same animal, cells with spikes of long duration and others with spikes of normal adult duration were found in rabbits less than two weeks old

Mean discharge rate

The mean discharge rate was measured for 351 cells When all the recordings were combined, the means and standard deviations of the mean impulse frequency (*F*) were computed for the different age

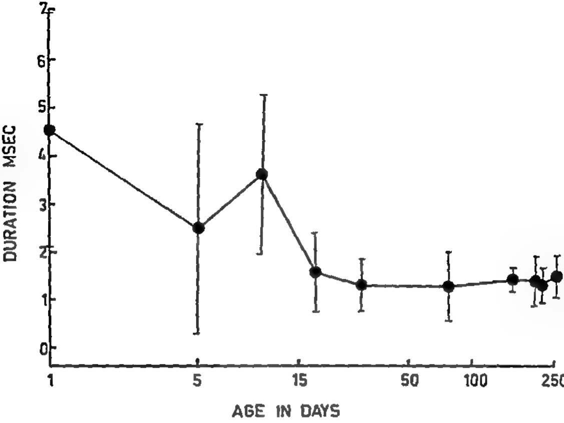


Fig. 4 The means and standard deviations of the duration of the extracellular action potentials in different age groups. The age is presented on logarithmic scale

groups These are to be found in Fig 5 During the first two postnatal weeks, the mean discharge rate was low 1 4 impulses per second for 0–2 days 1 7 for 4–6 days and 3 3 for 7–13 days of age On the 14th day of age a more marked change was apparent on examination of the individual values of *F* Thereafter, there continues a steady increase in the mean discharge rate until the age of two and a half months, which is for the rabbit the beginning of puberty (SANDFORD et al 1957) At this time the mean impulse frequency reaches a maximum of 18 6 impulses per second The next recordings are in the juvenile age after puberty and young adult rabbits at the age of five to eight months The mean discharge rate then diminishes considerably, first to the value of 10 4 imp sec but gradually increases again to 13 4 imp /sec at eight

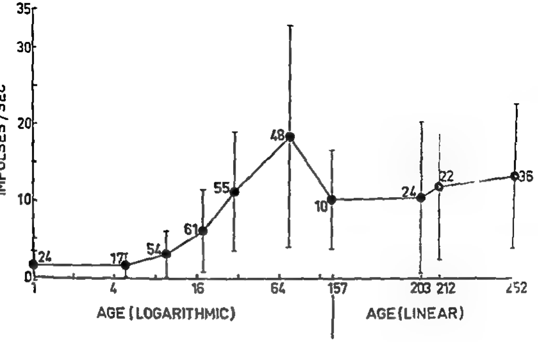


Fig. 5 The means and standard deviations of mean discharge rate (F) in different age groups in the whole material Age is given in days The figures indicate the number of cells in each age group For the growing up period a logarithmic time scale is used For time after puberty the scale is linear

months The dispersions of mean frequencies are great, a reflection of the fact that cells with very different mean discharge rates were met in the same animal

The individual values of F in different cells were plotted, with age in the abscissa The whole material was divided into age groups by examining variations in this plot Altogether, ten age groups were formed in this way These were 0–2 4–6, 7–13 14–22 26–33 69–84 157, 199–207, 212–213 and 249–255 days of age The distances between these groups and the numbers of cells in them are unequal but the mean discharge rates in each group form a more homogeneous set than that obtained on forming the age groups in other ways In many analyses, these age groups have been combined into wider groups

A study of the differences in the mean discharge rate between the examined diencephalic regions was made by means of an analysis of

variance As is indicated in Fig 5, the dispersions of F are great, and roughly proportional to the values of F in different age groups Consequently, there was effected a transformation to $\sqrt{F}$, which was justified by the similar relation of the means of F and $\sqrt{F}$ with respect to age or regions The samples from five regions in which most recordings had been made, were examined in three age group , as indicated in Table 4, where are presented the means of $\sqrt{F}$ and the numbers of ca es Age groups of less than fourteen days were not included, in view of the small number of observations in them in each region (cf Table 2) Consequently 12 groups were studied, as no recordings from *VA* and *VL* were made in the second age group, and none from *GP* in the third It should be noticed that the material in this anal sis was essentially divergent

TABLE 4

Means of $\sqrt{F}$ in different regions and age groups

REGION		AGE days	14–33	69–[illegible]	1[illegible]7–25[illegible]	Marginal
GP	mean		2.79[illegible]	4[illegible]94		4 094
			[illegible]	[illegible]		1[illegible]
CI	mean		2.[illegible]	[illegible]31[illegible]	4.2[illegible]6	3 733
	N		23	10	4	37
P	mean		2.972	3[illegible]	3 314	3 181
	N		29	14	15	[illegible]8
VA	mean		2[illegible]		2 91[illegible]	2.427
	N		10		16	26
VL	mean		1 427		3 671	3 030
					19	26
Marginal mean			[illegible]	4 4[illegible]1	[illegible]	3.2[illegible]
			[illegible]	[illegible]3	[illegible]4	1[illegible]2

The [illegible] variance within the groups s_w^2 1 73 3 with 1[illegible]0 degrees of freedom

from that presented in Fig 5 Only the differences in and ... th sides of puberty were investigated here

The equality of variances of $\sqrt{\bar{F}}$ was tested by means of B... t s test (HALD 1952, pp 290—293), and the resulting chi square ... 16 28 with 11 degrees of freedom was non significant Consequ... ailed analysis was made of the means of $\sqrt{\bar{F}}$ For convenience t... ter- action and effect have been adopted from analysis of varia... ol ogy These refer to differences between the groups studied ... not implied that these differences were due to any causal factors ... n (denoted by age X region) is said to be present if the effect of ... between regions To test for interaction between age and regi... s RAO s (1965, pp 211—214) method was applied The resul... ie computations are given in Table 5 By reason of the vigorous int... present, the effects of age have to be studied separately within ... region, and the effects of regions within each age group Tabl... shows the existence of significant differences in $\sqrt{\bar{F}}$ between regions in all age groups Table 6 B shows that the effect of age on $\sqrt{F}$ is ... nt in all regions except the thalamic non specific nuclei R and L 4 In ... n event, when examination was made of the points in the plot for F in the non specific nuclei for rabbits younger than 14 days there was noticeable an increase in F between 0 and 14 days It may be seen from table 4 that there is also a small increase in $\sqrt{\bar{F}}$ at puberty in R although this is less than in other regions

TABLE 5

Analysis of variance for the effects of age and region on $\sqrt{\bar{F}}$ (SS – sum of squares df degrees of freedom)

	SS due to unadjusted differences	SS due to adjusted differences	df
Age	66 014	53 830	2
Region	37 374	25 190	4
Age X Region	30 443	30 443	5
Between groups	121 647	121 647	11

The interaction is tested with F statistic $\frac{30\ 443}{5}$ — 1 3303 = 4 577 a significant F value at 0 1 level for (5 150) df

TABLE 6

A. Regional effects on $\sqrt{F}$ in different age groups

AGE days	df	SS	s_r^2	s_r^2 / s_w^2
14–33	4	14 594	3 649	2 743
69–84	2	20.260	10 130	7 615
157–255	3	20 778	6 929	5 209

All variance ratios are significant

B. Age effects on $\sqrt{F}$ in different regions

REGION	df	SS	s_a^2	s_a^2 / s_w^2
GP	1	14 410	14 410	10 832
CI	2	40 652	20 326	15 279
R + VA	2	5 944	2 972	2 234
VL	1	24 648	24 648	18 528

The variance ratio in R + VA is non-significant the others are highly significant
In all seven analyses of variance the estimate of within group variance $s_w^2 = 1\ 3303$ with 150 df was used.

STATISTICAL ANALYSIS OF PULSE TRAINS

There was a wide variation in the structure of the impulse sequences between cells Some examples of recorded pulse trains are to be found in Fig 6 Fig 7 illustrates some of the photographic point presentations of impulse sequences

The pulse trains were statistically analysed first by the study of interval histograms which characterize the occurrence of different lengths of intervals in the sequences Secondly, the autocorrelations were computed to reveal dependences between intervals In addition the probability of impulse occurrence in time was studied by computation of the intensity functions and renewal densities Examples of profitable use of these methods in the analysis of neuronal variability have appeared in the literature cited in the survey above

All these analyses were programmed and performed on the digitized data with a computer Brief accounts of the methods used for these analyses are given before the corresponding results

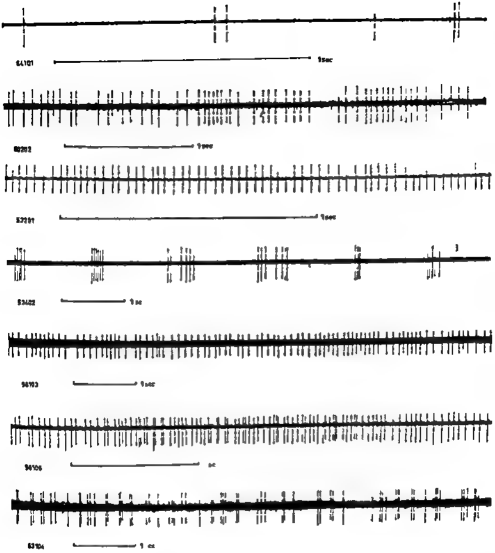


Fig 6 Pulse trains from different cells The impulse intervals from cell 64101 (recorded in VA of a 7 month-old rabbit) vary greatly Cell 60202 (ZI 6 months) gave a typical histogram Cell 53201 (CI 76 days) shows regular intervals Cell 53402 (R 76 days) fires in bursts Cell 58103 (R [illegible] months) gave a 6-modal histogram Cell 56106 (VL 8 months) shows a gradual change from long intervals to short ones and to long ones again Cell 63104 (Hp 7 months) gave a highly periodic intensity function

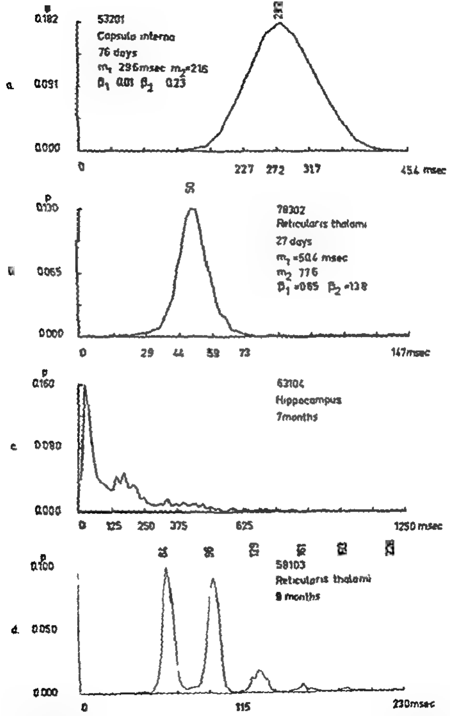


Fig. 10 Histograms a) A nearly normal distribution of 3255 intervals F = 33.8 b) A symmetrical leptokurtic histogram of 2994 intervals F = 19.4 c) A tri modal histogram of 1333 intervals F = 8.3 d) A six modal histogram of 3898 intervals F = 11.4

modes and four smaller ones are to be seen The impulse interval sequence of this neuron is shown in Fig 6, and the corresponding interval point presentation in Fig 7 The presence of several modes is also detectable in the point presentation The intervals between the consecutive modes are equal, and one half of the distance of the first mode from zero

The gamma distribution is the Pearson type III curve, its limit form is the normal distribution PEARSON and HARTLEY (1956) have presented the relationship between skewness and kurtosis for different Pearson curves For gamma distributions, the relationship between β_1 and β_2 is linear The line which relates to this relationship is indicated in Fig 11, in which are plotted the (β_1, β_2) pairs for all the distributions Most of

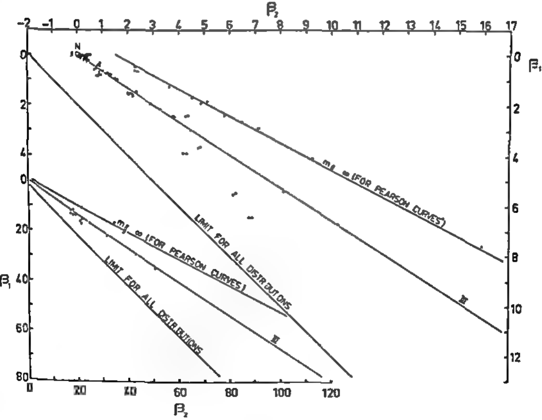


Fig 11 The $(\beta_1\ \beta_2)$ pairs for different samples The smaller graph to the left gives the continuation of the figure showing pairs with greater values There are observable the lines representing the limit for all distributions and $m_4 = \infty$ for Pearson curves and the Pearson type III line representing the gamma distribution The normal distribution (N) is located in the upper end of the type III line

the points differ from the type III line, although 14 points are very close to it, thus on the basis of their first four moments 14 distributions could be of gamma type. When these 14 histograms were examined more closely, it appeared that six of them had too high a peak and too long a tail for gamma distribution. Eight of these 14 could be gamma distributions on the basis of their appearance also.

No systematic association of exponential histograms was found in respect of brain area or age of the experimental animal. Against this, in the internal capsule, globus pallidus and zona incerta, the proportion of nearly normal distributions was much higher than in other regions, as can be observed in Table 7.

TABLE 7

Occurrence of bell-shaped distributions in different regions

REGION	GP CI ZI		Others		Total	
	%	N	%	N	%	N
Bell shaped	24	17	5	9	11	26
Others	76	54	95	164	89	218
Total		71		173		244

Chi-square 18.10 df 1 Significant at 0.01 level

The multimodal distributions were commoner in higher age groups and in the non specific thalamic regions (Table 8). In this respect also the nucleus lateralis anterior resembled the non specific nuclei.

Six of the seven symmetrical but more peaked distributions than the normal were found in the nucleus reticularis thalami of two rabbits aged 26 and 27 days. The seventh was found in the globus pallidus of a 30 day-old rabbit. All these extremely regularly firing cells were thus recorded in the same age group of animals approximately one month old, and with one exception all in the thalamic reticular nucleus.

TABLE 8

A Occurrence of multimodal distributions in different age groups

AGE days	0—13		14—81		157—255		Total	
	%	N	%	N	%	N	%	N
Multimodal	0	0	10	16	18	16	11	32
Others	100	53	90	140	82	73	89	266
Total		53		156		89		298

Chi-square = 11.21 df = 3 Significant at 0.05 level

B Occurrence of multimodal distributions in different regions

REGION	R VA LA		Others		Total	
	%	N	%	N	%	N
Multimodal	19	19	8	11	12	30
Others	81	83	92	131	88	214
Total		102		142		244

Chi-square = 6.62 df = 1 Significant at 0.05 level

Autocorrelations

Estimates for the autocorrelations of the interval sequences of each sample were computed from the whole sample if this included less than 500 intervals, otherwise the 500 first intervals were used. The autocorrelations were computed for all the samples with lags of 1—6 and denoted by AC_1—AC_6. In a number of cases where some of these were relatively high additional autocorrelations were computed with lags of 7—50.

Fig 12 presents some autocorrelations. For cell number 60202, the corresponding histogram is given in Fig 8 a). In this rather typical case, the first autocorrelation is 0.17 and thereafter the autocorrelation values fluctuate around zero. It is notable that the ampitude of these fluctuations does not diminish with increasing lag but that they are still distinguished up to lags of 40—50.

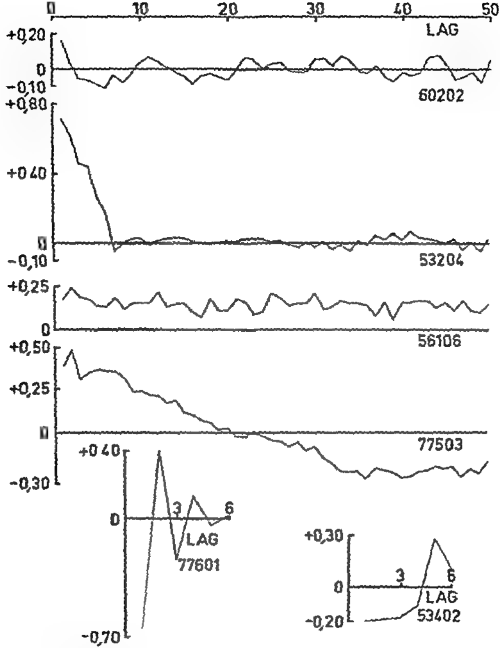


Fig. 12. Autocorrelation sequences from different cells. The coding numbers of the cells, corresponding brain regions and ages of the experimental animals are given here: 60202 ZI, 6 months; 53204 R, 6 days; 56106 VL, 8 months; 77503 R, 26 days; 77601 R, 26 days; 53402 R, 26 days.

The first autocorrelations of cell 53204 have very high values ($AC_1 = 0.724$) but from lag 7 onward the values are nearly zero. Here also they display a slightly cyclic tendency to stay for a while on one side of the zero line.

The pulse train from cell 56106 is shown in Fig. 6. The corresponding photographic points exhibited a gradual cyclically repeated shift from short to long intervals and to short ones again. One such cycle from succeeding short intervals to succeeding long intervals apparently contained on the average more than 50 intervals. If autocorrelations with greater lags had been computed, perhaps, a change to negative autocorrelations would have appeared before the whole cycle had passed from succeeding short intervals to succeeding long intervals.

The photographic point presentation for neuron 77503 is reproduced in Fig. 7. As is observable in that figure, this neuron cyclically repeated a gradual change from succeeding short intervals to succeeding long ones. This phenomenon is the same as that which occurred in neuron 56106, but the cyclic frequency was faster in neuron 77503. The autocorrelations of neuron 77503 consequently evince a change from significantly positive to significantly negative values.

Neuron 77601 in the same animal fired in sequences, a short interval being nearly always followed by a long one, and a long one by a short (cf. Fig. 7). The autocorrelation values gradually approach zero, which indicates that the interval lengths have a random variability which attenuates the correlation when more intervals intervene. The corresponding histogram was bimodal, with a high peak in the first class, and the distribution around the second mode was bell shaped.

Neuron 53402 was recorded in the animal which also gave the second example of autocorrelations. The first four autocorrelations are significantly negative, and the fifth and sixth are positive. The distribution was a bimodal one, in which the second mode was rather flat. The corresponding pulse train is presented in Fig. 6; in this figure it is observable that this cell fired in bursts, which contained 4—7 impulses. As the intervals between the bursts are longer than those inside the bursts, the first four autocorrelations are negative. In any event the sequence repeats itself rather regularly, and accordingly the fifth and sixth autocorrelations are positive.

The first six autocorrelations, determined in all the samples, exhibited extensive variation, illustrated by the examples presented in Fig. 12. It was not possible in any way to relate this variation to the age of the experimental animal, the brain regions, the forms of the histograms, or the function of cells displaying a tendency towards bursts of spikes.

However, one tendency existed in the autocorrelation values, the autocorrelations with different lags inclined to vary in unison. Thus they all tended to be positive, sometimes with high values, or they all tended to be negative, or all nearly zero. There were clear deviations from this principle, but broadly speaking this tendency was discernible. With all lags, positive autocorrelation was more frequent than was negative. In conjunction with the multivariate analysis, described below (cf. p. 56), however, some general features of the variation in the autocorrelation values became evident.

Intensity functions and renewal densities

The time structure of impulse sequences can be studied in terms of the probability of impulse occurrence as a function of time. This function has also been entitled expectation density (POGGIO and VIERNSTEIN 1964) and intensity function (COX 1965). In this context the term intensity function will be employed. The sequence of all or none type impulses can be regarded as a point process, i.e. as a collection of separate events taking place at particular points of time. According to COX (1965) the intensity function $g(x)$ of a stationary point process gives as a function of x the conditional probability density, given an event at time t, of an event in the interval $(t+x, t+x+\Delta x)$. Thus for $x > 0$,

$$g(x) = \lim_{\Delta \to 0} \frac{\text{prob}\{\text{event in } (t+x, t+x+\Delta x) \mid \text{event at } t\}}{\Delta x}$$

In the theory of stationary point processes, a special position is occupied by *renewal processes* (see COX 1962, p. 25). These are characterized by 1) all intervals between events following the same distribution and 2) the intervals being independent stochastic variables. This means that the past history of the process exercises no effect on the length of any particular interval. In the case of a renewal process, the intensity function, called the renewal density (COX 1965) and denoted by $u(x)$, is determined by the distribution of interval lengths $f(x)$ according to the integral equation of renewal theory

$$u(x) = f(x) + \int_0^x u(y) f(x-y)\,dy \quad \text{(COX 1962, p. 54)}$$

The *Poisson process* is a special renewal process, with exponential distribution of interval lengths For a Poisson process, the intensity function has a constant value, which means that there is an equal probability of an event at any particular time after the start of the process

In this study, the intensity functions were estimated for all 298 samples In addition, the renewal densities were computed on the basis of the observed distributions of interval lengths If an observed process is a renewal process the intensity function and the renewal density should be close to each other The calculated renewal density corresponds to the intensity function obtained from shuffled data (POGGIO and VIERNSTEIN 1964) The discrepancies between the intensity function and the renewal density can be used to judge the presence of dependences between intervals (= time dependence)

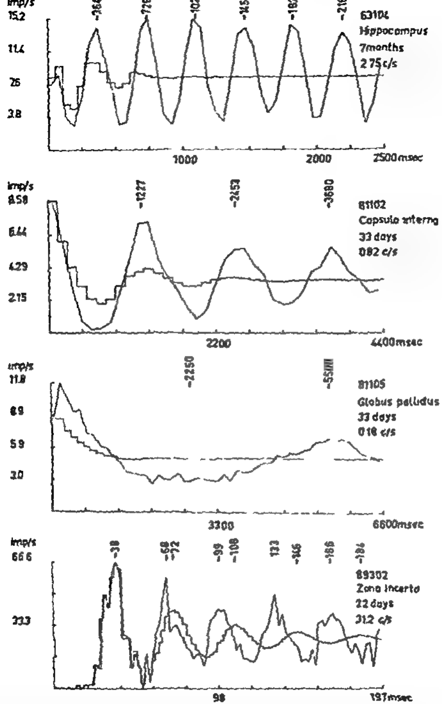
For computing the estimate of the intensity function (*IF*) all pairs of events are considered and a frequency histogram is made of intervals between them The same grouping interval (h) was employed as in the formation of interval histograms (cf page 31) For more detailed reference to the estimation of *IF* see Cox (1965) As the computation of *IF* for all the samples would have required lengthy periods of computer time the *IF* s were computed for samples of at most 300 or 500 intervals The number of classes N_{IF} for which the *IF* was estimated constituted a multiple of the number of classes in the interval histogram kN_H where $k = 2$ 4 6 or 10 was used

The values of the renewal density (*RD*) were computed on the basis of the observed interval histogram The computations were based on the integral equation by assuming a constant value for $u(y)$ within small grouping intervals

The *IF* and *RD* were derived by means of the computer tabulated together and drawn by a digital plotter on a standard scale The *IF* was drawn in the form of a frequency polygon A bar graph was employed for *RD*

The present material was found to contain many different types of intensity functions and renewal densities Some typical examples of the different types will be shown Data relating to the frequency of occurrence of these types is presented later

Fig 13 a) shows the *IF* and the *RD* of cell 63104 The corresponding interval histogram appeared in Fig 10 c) and the pulse train in Fig 8 The *IF* shows a highly periodic character, even following stabilization of the *RD* No signs of reduction of the amplitude are discernible The mean interval for this neuron was 155 msec, and the corresponding mean frequency of firing 6 44 impulses per second The duration of one intensity cycle is 364 msec, and the corresponding cyclic frequency of intensity fluctuation 2 75 cycles per second The peaks of *IF* amount to 15 impulses per second, whereas the *IF* minima are at a level of 3 impulses per second The level at which the *RD* has stabilized is 8 34 which is the mean frequency of firing On the basis of the interval lengths alone without



g. 13 Periodical intensity functions (IF) drawn as frequency polygons and associated renewal densities (RD) drawn as bar graphs. The IF gives the probability of impulse occurrence in time in impulses per second. The RD gives this probability if there is no dependence between intervals. The differences between these curves can be used for judgement of the presence of dependence between intervals. The mean impulse frequency (F) heart

any time dependent order of the succeeding intervals, a small variation in the likelihood of impulses is noticeable during three intensity cycles, but disappears after this

Fig 13 b) presents another periodic intensity function The corr spond ing histogram was bimodal The mean frequency of firing is 3 (pulses per second, and the mean interval 277 msec The periodic intensity fluctuation exhibits a frequency of 0 82 cycles per second, and one cycle lasts 1,227 msec The amplitude of the oscillations diminishes with increasing time This type of slightly waning periodic intensity fluctuation is the same as that found by POGGIO and VIERNSTEIN (1964) in unanaes thetized monkeys, in 20 thalamic somatic sensory neurons of a selected material of 26 neurons In any case the intensity cycle is considerably longer

Fig 13 c) presents an even more slowly oscillating intensity function The $m_1 = 195$ msec, $F = 5\ 13$ The intensity cycle is 5 5 sec, and corresponds to a cyclic frequency of 0 18 c/s

Fig 13 d) shows a third type of periodic *IF* This unit also presented a bimodal histogram, with the $m_1 = 40\ 47$ msec, $MO = 37\ 80$ msec and $F = 24\ 73$ imp /sec The *IF* and *RD* copy the histogram in the first intensity cycle, and then shift apart from each other The mean period of the *RD* is 36 80 msec, which is near the value of the mode The mean cycle of the *IF* is 33 20 msec The reason for this phase shift is not known Computation of the *RD* from grouped data may introduce slight errors in the exact amount of the phase shift, but cannot serve to explain a phase shift of 3 60 msec per period the value in this instance

However there are other cases in which such a phase shift could be interpreted as an artefact attributable to the method of computing the *RD* from grouped data In Fig 14 a) such a case is presented The corresponding histogram is symmetrical with $\beta_1 = -0\ 018$ but leptokurtic with $\beta_2 = 4\ 177$ The coefficient of variation is 7 49 per cent Thus the pulse train is very regular and the variation in the intervals

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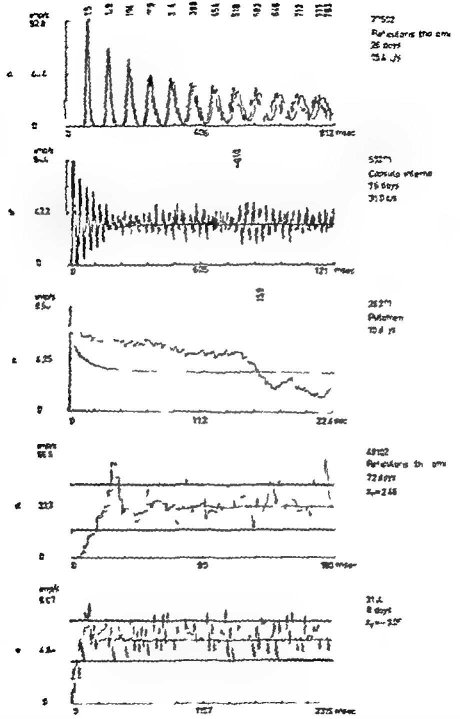
rate and ventilation rate are given below The sample size used was 300 for all the samples in this figure

Cell number	F imp /sec	Heart rate /min	Ventilation rate /min.
63104	8 3	330	80
81102	3 6	375	110
81105	5 1	400	80
89302	24 7	375	70



F[illegible] 11. In[illegible] functions and renewal densities. The sample size was [illegible] in a and b, and 3[illegible] in the other samples. In d and e the horizontal lines indicate the significance limits for individual values of IH at 0.05 level under the null hypothesis of a Poisson process. Number of classes N_{H} was in d [illegible]80 and in e [illegible]119. In e the histological localization was unsuccessful.

small In such cases, the *RD* should be strictly periodic (Cox 1962, p 55) with maxima near m_1, $2m_1$, and minima near 0, $\frac{3}{2}m_1$, $\frac{5}{2}m_1$, and by virtue of the small dispersion the oscillations should continue for a considerable time In this cell 77502, m_1 = 65 00, *MO* = 65 18 msec and F = 15 40 imp /sec The *IF* is very highly periodic, as is the *RD* Thus there exists no time dependence between the intervals i e this highly periodic process is a renewal process, in which the periodicity is wholly caused by the almost equal lengths in the intervals and not at all by the sequential order of the intervals The cyclic frequency of the intensity fluctuation is 15 40 the same as the mean impulse frequency There is also a phase shift which amounts to 6 48 msec for 12 intensity cycles The phase shift is consequently 0 54 msec per period There is a difference between the mean and the midpoint of the class containing the mean of 0 88 msec, this is more than the phase shift Thus in this case the phase shift is an artefact attributable to the method of estimating the *RD*

There were furthermore some cases of modulation of the intensity cycles The *IF* for cell 53201 is presented in Fig 14 b) The corresponding interval distribution is to be found in Fig 10 a) This was a normal distribution It is observable that both the *IF* and the *RD* oscillate at a cyclic frequency of 31 c/s, but the *PD* soon stabilizes to the mean frequency However, the *IF* recommences the oscillations after about 910 msec with the same cyclic frequency

Some of the intensity functions evinced deviations from the renewal density which were not periodic Fig 14 c) illustrates the *IF* of cell 26201 For about 16 seconds there is a constantly high probability of firing then this probability drops suddenly When the photographic interval spots of this sample shown in Fig 7, are examined with reference to this phenomenon, they reveal long bursts of spikes The duration of the bursts varies from 1 5 to 30 seconds the mean possibly being of the order of 16 seconds

Two different measures were adopted to test the possible non periodic time dependence as reflected by the *IF* As has been indicated by Cox (1965) when the *IF* tends to a limit with increasing x, this limit will be F For a Poisson process the *IF* should be nearly constant and have the value of F for all x The null hypothesis of a Poisson process permits of the construction of significance limits for individual values These have been given for the 5 per cent limits by Cox and Lewis (1966 p 123)

Fig 14 d) shows the *IF* of cell 49102 The significance limits quoted above for individual values of *IF* under the null hypothesis of a Poisson process have been incorporated in the figure As the *RD* is not a constant

line from the beginning, and indicates that the process is not Poissonian, these limits should, with a view to testing deviation from a renewal process, be valid only when the *RD* has stabilized, which happens approximately at the middle of the estimated interval. Thereafter the *IF* exceeds these limits in six of the 40 classes. As the limits are computed for individual values at the 5 per cent significance level, no more than two values outside the limits could be expected at this level of significance. This neuron accordingly exhibits time dependence which is of a non-periodic nature.

Another measure of significant differences between the *IF* and the *RD* was constructed on the basis of the theory of runs up and down. This was done primarily for the multivariate analysis described later. The run test measures how often the *IF* crosses the *RD* and whether the number of crossings deviates from what could be expected on a purely random basis. The number of crossings was transformed into a unit normal deviate z_r (SIEGEL 1956, pp. 52—58). Large negative values of z_r correspond to an excess of crossings, and large positive values to few crossings.

For neuron 49102 (Fig. 14 d) the value of z_r is 2.46, which is significant at the 5 per cent level. Thus this measure also shows time dependence, which is apparently of non periodic nature.

Negative significant values of z_r were occasionally found as well, which would indicate that the *IF* crosses the *RD* too often for this crossing to happen at random. Such an example is illustrated in Fig. 14 e). The *IF* is seen frequently to cross the quickly stabilized *RD*. Moreover, the fluctuation of the *IF* often exceeds the 5 per cent significance limits. The value of z_r is -3.05, which is significant at the one per cent level. It is not known whether this phenomenon is of any physiological importance.

A study was made of the frequency of occurrence of periodic intensity functions and renewal densities in the brain regions and age groups subjected to examination. In the whole material, which consists of 298 analysed *IF*'s and *RD*'s, there were 52 *IF*'s in which cyclic intensity fluctuation was discernible. Examples of such periodic *IF*'s are to be seen in Fig. 13. In this group, the *IF* differed clearly from the *RD* either in it being periodic, such periodicity not being present in the *RD*, or in the amplitude of the intensity oscillations being greater in the *IF* than in the *RD*. In the whole material this type of *IF*, referred to below as periodic *IF*, was found in 18 per cent of the samples analysed. In an additional 34 samples (11 per cent), the run test gave significant z_r-values indicating indefinite periodic or non periodic time dependence, as illustrated in Figs. 14 d) and e). In the remaining 71 per cent, no

Table 9

Occurrence of periodic intensity functions in different age groups

AGE days	0–6		7–33		69–84		[illegible]		tal
	%	N	%	N	%	N			N
Periodic	0	[illegible]	14	20	30	14	20	18	[illegible]
Non periodic	100	20	86	127	70	28	80	1	[illegible]
Total		20		147		42		89	

Chi square = 13 56 df = 3 Significant at 0 01 level

time dependence was found In these cells the pulse train can be [illegible] as a renewal process, in which the sequential order of the [illegible] not of importance

The *RD* demonstrated periodic behaviour in 51 samples (17 per [illegible] in which the *RD* and *IF* followed an equal time course Thus [illegible] appearance of the *IF* s in these samples was wholly related to the [illegible] bution of the interval lengths These cases will be referred to as peri[illegible] *RD* s In such instances, the distribution has a relatively small dispersion which leads to periodic renewal density

When the frequency of occurrence of periodic *IF* s was examined in different age groups highly significant differences were found as is illustrated in Table 9 The age distribution of the periodic *IF* s is shown in Fig 15 as a percentage of samples analysed The periodic time dependence increased sharply in two age groups — at the age of 6–13 days and at the beginning of puberty, at the age of two and a half months At puberty, the periodic time dependence exceeded the later value A gradual increase, toward adult values in the periodic time dependence was also observable in the juvenile groups after puberty

The periodic *IF* s were examined in different brain regions The corresponding contingency table is given in Table 10 A As in this case no rational grounds were discoverable for the combination of regions the 11 regions were studied as such, even if at times the expected number of periodic *IF* s was low (minimum 1 74) This may introduce a slight bias in the chi square, tending slightly to increase the chi square value Despite this, the computed value of 8 07 is clearly non significant with the 10 degrees of freedom Accordingly, no significant differences are

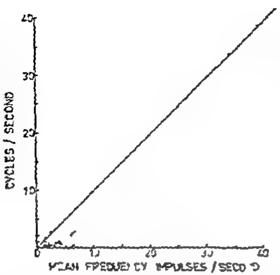


Fig. 15. The relation between mean impulse frequency and cyclic frequency of intensity fluctuation. The line at an angle of 45° represents the equality of these frequencies. With high mean frequency many points fall on this line. With low mean frequency the cyclic frequency is generally slower than the mean frequency. The cases considerably above this line show multimodal distributions.

TABLE 11

Observations of periodic intensity fluctuation (cycles per second)

Cycles/second	Number of cases (Total 52)
0.05 – 0.50	16
0.51 – 1.50	10
1.51 – 3.00	7
3.01 – 5.00	2
5.01 – 10.0	1
10.1 – 20.0	3
20.1 – 40.0	12

though the amplitude of oscillations was greater in the *IF*. The greater amplitude of the *IF* indicated a tendency to correct the values of the intervals in such a way that when an interval was longer than the mean interval, the following one tended to correct this, and to be shorter than the mean. The frequency of firing was then equal to the cyclic frequency. The ratio of the cyclic frequency to the mean frequency was one for all the 51 periodic *PD*s as well.

No correlation with age or the brain regions examined was found for the cyclic frequency or its ratio to *F*.

A measure of the deviation of the *IF* from the constant line of the *IF* for a Poisson process was formed of the chi-square (χ^2) between the *IF* and this line (Cox, 1965). The chi-square values obtained were nearly always highly significant, which indicated that the *IF* was very seldom that of a Poisson process. Only 13 non-significant chi-square values at the 0.01 level were found. Of the possible Poisson processes, three were recorded in adult rabbits and one at the age of one month. The others were recorded in rabbits less than three weeks old. None of the regions studied was excessively represented among these cells.

The highly significant values of the chi-square were probably often related to the form of the *IF* following the histogram during the gradual rising phase (Fig. 14 d).

ASSOCIATIONS BETWEEN DIFFERENT MEASURES OF NEURONAL ACTIVITY

With the multitude of results recorded, it was evident that efforts should be made to combine the different findings This was done first by analysing the correlations between different measurements obtained from each cell, and secondly by applying the methods of principal component and factor analysis to these correlations Finally principal component scores were computed for each cell, and related to the brain region and to age

Correlation matrix

First of all the variables used in these studies will be defined Most of them have been met earlier, athough some transformations have been introduced to give a more linear character to the relationships between variables

I General measurements

1 *Age* of the experimental animal in days Age from 0 to 24 hours was designated as one day and from 25 to 48 hours as one and a half days Otherwise full days were used

2 *Log age*

3 *Duration* of the extracellularly recorded action potential in milliseconds (cf p 22)

4 *Amplitude* of the action potential peak to peak This measure is obviously rather crude as it depends on the distance of the recording electrode from the cell As in any case there were no disadvantages attached to accepting many variables in the correlation matrix this measure was taken into use As will be shown this did not result in high correlations with any variables other than the duration of the action potential

5 *Resistance* of the recording electrode in megohms (cf p 15) this was accepted with a view to determining whether the tip size of the electrode plays any role possibly in the selection of cells different in size and form of action It was found that this was not correlated with any other variables

6 *Distance* to the nearest other spontaneously active cell along the same electrode track (cf p 21) This did not evidence high correlations

II Variables obtained from histogram

7 Mean discharge rate (F) (cf p 31)

8 The square root $\sqrt{F}$ (cf p 26)

9 Mean interval (m_1) (cf p 31)

10 Coefficient of variation (c) given by $c = \frac{\sqrt{m_2}}{m_1}$ (cf p 31 for m_1 and m_2) which measures the dispersion of the histogram

11 The mode (MO) of the histogram (cf p 31)

12 The median (MED) of the histogram (cf p 31)

13 As a measure of *skewness* (cf p 31) $\log(\beta_1 + \frac{1}{2})$ was used The logarithmic transformation was effected in order to diminish the very high dispersion of β_1 values in this material (cf Fig 11)

14 As a measure of *kurtosis* (cf p 31) $\log(\beta_2 + 2\frac{1}{2})$ was used on grounds similar to the above (cf Fig 11)

III Variables related to time dependence

15 – 20 The first six autocorrelations $AC_1 - AC_6$ (cf p 37)

21 As a measure of the deviation of the *IF* observed from Poissonian constant value *IF*, the variable *P* defined by

$$P = \log\left(\frac{\gamma^* - \lambda_{IF}}{\sqrt{2\lambda_{IF}}} + 1\right)$$ was used (for γ cf p 50) This logarithmic transformation

was introduced to diminish the very high dispersion of the values of this variable

22 As a measure of deviation of *IF* from *RD* use was made of the variable z_r (cf p 46)

Adequate description of the deviation of the *IF* from *RD* and Poissonian *IF* proved to be a difficult task It may be mentioned that other measures were also tried but these had to be deleted from the final analysis as they gave counter intuitive results As a check correlations of all the time dependence measures with nuisance parameters sample size number of classes for *IF* and the length of grouping interval were examined All the measures showed a considerable degree of correlation with the nuisance parameters

The correlation matrix obtained is presented in Table 12 As 298 samples are included in this analysis, the significance limits for deviations from zero are given by $\pm 0\,11$ at the 0 05 level, and $\pm 0\,15$ at the 0 01 level

The correlations between the different transformations of the same variable, such as *F* and $\sqrt{F}$ are obviously high, but of no interest Study of the correlations of such parallel indicators with the other variables led to selection of the one showing greater values for the multivariate analysis The values of $\sqrt{F}$ are usually higher than those of *F*, and thus $\sqrt{F}$ was accepted

Brief comments on the findings of the correlation analysis are presented below *The logarithm of the age shows a high negative correlation* (−0 50) with the duration of the action potential This is apparent in Fig 4 The age shows a definite correlation with the *F* and its parallel indicators $\sqrt{F}$ m_1 *MO* and *MED* It is interesting to note that age evidences no correlation with the coefficient of variation (*c*), or with skewness or kurtosis

The duration of the action potential displays a high correlation with the amplitude of the action potential, this might bear some relation to the phenomenon of giant extracellular responses referred to on p 22 Long duration correlates with Poissonian tendency (= negatively with *P*)

Table 12

Correlation matrix

	age	log age	duration	amplitude	resistance	distance	F	$\sqrt{F}$	m_1	c	MO	MED	skewness	kurtosis	AC_1	AC_2	AC_3	AC_4	AC_5	AC_6	P	z_r
age																						
log age	09																					
duration	−30	−50																				
amplitude	15	07	26																			
resistance	05	10	00	04																		
distance	−12	−11	−03	03	−03																	
F	25	38	−29	01	08	−08																
$\sqrt{F}$	29	43	−36	−01	05	−09	97															
m_1	−14	−24	23	10	14	03	−39	−53														
c	03	−01	15	13	−05	11	−47	−52	25													
MO	−25	−00	31	03	01	01	−32	−43	[illegible]	00												
MED	−15	−25	24	08	15	02	−36	−48	97	11	82											
skewness	02	−04	15	09	−03	18	−35	−35	14	63	06	07										
kurtosis	00	−05	11	00	−02	17	−20	−19	00	46	02	00	92									
AC_1	05	01	−01	04	−06	−13	10	14	−06	−19	04	−04	10	18								
AC_2	−04	−06	00	−04	02	−12	17	14	12	−27	16	19	−17	−10	60							
AC_3	−09	−13	10	07	01	01	08	04	06	15	13	07	04	03	56	62						
AC_4	−19	−21	00	01	07	−05	10	00	05	−16	12	08	−09	−04	30	50	58					
AC_5	−15	−18	18	07	02	−03	−08	−11	04	−03	12	06	−01	01	30	39	52	53				
AC_6	−14	−14	10	06	13	05	04	00	09	−11	10	11	−00	02	15	31	43	40	50			
P	01	11	−26	−01	−03	−01	18	20	−34	21	−42	−14	−07	−01	−11	12	−11	−05	−07	−02		
z_r	01	05	−13	−01	−04	08	06	06	−06	28	−13	−12	07	12	−08	−03	−07	−05	−08	−03	54	

13 As a measure of *skewness* (cf p 31) log $(\beta_1 + \frac{1}{2})$ was used The logarithmic transformation was effected in order to diminish the very high dispersion of β_1 values in this material (cf Fig 11)

14 As a measure of *kurtosis* (cf p 31) log $(\beta_2 + 2\frac{1}{2})$ was used on grounds similar to the above (cf Fig 11)

III Variables related to time dependence

15 – 20 The first six autocorrelations $AC_1 - AC_6$ (cf p 37)

21 As a measure of the deviation of the *IF* observed from Poissonian constant value *IF* the variable *P* defined by

$P = \log \left(\frac{\gamma^* - \lambda_{IF}}{\sqrt{2\lambda_{IF}}} + 1 \right)$ was used (for γ^* cf p 50) This logarithmic transformation was introduced to diminish the very high dispersion of the values of this variable

22 As a measure of deviation of *IF* from *RD* use was made of the variable z_r (cf p 46)

Adequate description of the deviation of the *IF* from *RD* and Poissonian *IF* proved to be a difficult task It may be mentioned that other measures were also tried but these had to be deleted from the final analysis as they gave counter intuitive results As a check correlations of all the time dependence measures with nuisance parameters (sample size number of classes for *IF* and the length of grouping interval were examined All the measures showed a considerable degree of correlation with the nuisance parameters

The correlation matrix obtained is presented in Table 12 As 298 samples are included in this analysis, the significance limits for deviations from zero are given by $\pm$ 0 11 at the 0 05 level, and $\pm$ 0 15 at the 0 01 level

The correlations between the different transformations of the same variable, such as *F* and $\sqrt{F}$ are obviously high, but of no interest Study of the correlations of such parallel indicators with the other variables led to selection of the one showing greater values for the multivariate analysis The values of $\sqrt{F}$ are usually higher than those of *F*, and thus $\sqrt{F}$ was accepted

Brief comments on the findings of the correlation analysis are presented below The logarithm of the age shows a high negative correlation (–0 50) with the duration of the action potential This is apparent in Fig 4 The age shows a definite correlation with the *Γ* and its parallel indicators $\sqrt{\Gamma}$, m_1, *MO* and *MED* It is interesting to note that age evidences no correlation with the coefficient of variation (*c*), or with skewness or kurtosis

The duration of the action potential displays a high correlation with the amplitude of the action potential, this might bear some relation to the phenomenon of giant extracellular responses referred to on p 22 Long duration correlates with Poissonian tendency (= negatively with *P*)

TABLE 13

Principal components

	1	2	3	4	5
EIGEN VALUE	3.54	2.77	1.99	1.34	1.05
VARIABLE					
duration	−0.12	0.45	0.40	0.23	0.23
distance	0.14	0.22	−0.10	0.12	−0.80
$\sqrt{F}$	−0.14	−0.66	−0.34	−0.33	−0.19
c	0.43	0.66	−0.25	0.21	0.21
MO	−0.23	0.38	0.58	0.12	0.10
skewness	0.27	0.84	−0.24	−0.32	−0.07
kurtosis	0.18	0.75	−0.34	−0.39	−0.12
AC_1	−0.61	0.14	−0.23	−0.55	0.19
AC_2	−0.78	−0.02	−0.14	−0.18	0.18
AC_3	−0.80	0.18	−0.19	−0.06	−0.01
AC_4	−0.74	0.11	−0.17	0.20	−0.05
AC_5	−0.69	0.26	−0.13	0.33	−0.05
AC_6	−0.61	0.16	−0.17	0.40	−0.28
P	0.25	−0.23	−0.72	0.33	0.19
Z_T	0.23	0.00	−0.65	0.31	0.24

TABLE 14

Varimax rotation

VARIABLE	1	2
$\sqrt{F}$	0.04	−0.57
c	−0.18	0.79
skewness	0.01	0.94
kurtosis	0.09	0.85
AC_1	0.66	0.03
AC_2	0.76	−0.21
AC_3	0.84	−0.04
AC_4	0.76	−0.09
AC_5	0.75	0.06
AC_6	0.65	−0.01

MO, *P* and z_r. Two principal components were extracted from the remaining variables, and a Varimax rotation carried out. The result is given in Table 14.

With these results as basis, two new combined variables were formed as weighted sums of the original ten variables, using the component loadings as weights. Thus, as is shown in Table 14, high values of the first combined variable are associated with positive values of the autocorrelations. Low values are associated with zero or negative autocorrelations. Similarly, high values of the second combined variable indicate high values of dispersion, skewness and kurtosis, and a low value of mean frequency. Low values indicate nearly normal distribution and high mean frequency.

Essentially, these results show that several characteristics of the histogram were so interrelated in the present material that they can be consistently combined into a single parameter, which describes the histogram. Similarly, the different autocorrelations do not seem to vary independently of each other. The information given by the first six autocorrelations can thus be combined to form another single parameter, which describes the correlations between intervals.

Principal component scores

The principal component scores of autocorrelation component and histogram component were computed for each cell. The means of these scores and their variances were calculated in different age groups, and for the regions and age groups presented in Table 4. One way analysis of variance was effected to examine the differences between age groups in terms of component scores. For the differences in autocorrelation component scores in different age groups this analysis of variance gave a significant value at the 0.05 level. The means of component scores are presented in Fig. 17. The autocorrelation values diminish slightly with increasing age although the greatest discrepancy lies between the lowest age group and the other groups. No special differences are discernible at puberty.

The result of a similar analysis carried out with respect to histogram component scores is significant at the 0.1 per cent level. As can be seen in Fig. 17 the differences in the histogram component scores between age groups are very similar to the differences in mean discharge rate (cf. Fig. 5). In low age groups the values of the combined histogram variable are high and indicate high values of dispersion, skewness and kurtosis, and a low discharge rate. A diminution in this value towards puberty indicates an increase in nearly normal distributions and dis

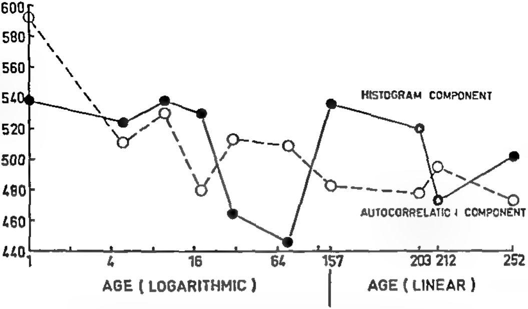


Fig 17 The means of principal component scores in different age groups Age is given in days The first or autocorrelation component is drawn with a dotted line and the second or histogram component with a solid line The analysis of variance indicated that the differences between the age groups were significant at the 0.05 level for the autocorrelation component and at 0.1 % level for the histogram component

charge rate After puberty, an increase is again discernible This is in accordance with the results obtained in terms of periodic *RD* s and *F* (cf Figs 5 and 15) The differences observed between the histograms in different age groups can thus be characterized by one single variable

A two way analysis of variance in respect of the differences between regions and age groups was made on the scores This was effected in a way similar to the analysis performed on $\sqrt{F}$ (cf p 27) The autocorrelation component scores evidenced no significant differences with respect to age or region The histogram component scores showed a highly significant age × region interaction ($p < 0.1$ per cent) This was, as in the analysis of $\sqrt{F}$ attributable to small differences between age groups in the thalamic non specific nuclei *R* and *VA* and large differences between age groups (low values at puberty) in the other regions, *GP* *CI* and *VL*

DISCUSSION

Considerations with respect to anaesthesia

It is known that anaesthetic agents lower the discharge rates of neurons (MOUNTCASTLE et al 1957, WERNER and MOUNTCASTLE 1963, ANGEL 1964) In the current study, the mean discharge rate and the frequency of intensity fluctuations were usually slower in adult rabbits than has been reported by POGGIO and VIERNSTEIN (1964) in respect of thalamic somatic sensory neurons in unanaesthetized adult monkeys The anaesthetic level was so regulated in this study that differences between age groups should not arise as a result of anaesthesia The initial dosage of urethan (Table 1) required for similar conditions of anaesthesia, as judged on the basis of the general state of the animal, the breathing rate, ECG, ECoG, and withdrawal reflex, was slightly larger in the age group at the beginning of puberty, interesting phenomena were remarked in this age group It is possible that this difference in the amount of urethan needed for similar conditions of anaesthesia in this age group is associated with the differences observed in the neuronal activity This association must, however, be quite complicated and indicate a change in the action of urethan in this age group Otherwise it should be borne in mind that it is probable that the anaesthesia influenced the general nature of the results

Extracellular action pontentials

The difference in the duration of the action potential, presented in Figs 3 and 4, is open to some criticism in view of the extracellular recording employed (WOODBURY 1961) During the progress of this work, intracellular recordings of long lasting action potentials have been reported in the neocortex of newborn kittens by PURPURA et al (1965) As the recording distances from the cell are of the order of 100 microns or so, the lengthening effect of distance on the action potential is very small The difference between age groups is on a firm basis, since the

recording was effected in the same way for all age groups

The so called giant extracellular responses recorded more frequently in young animals could possibly be interpreted as a kind of passive response that has been capacitatively coupled across the cell membrane to the recording electrode (FREYGANG and FRANK 1959, NAKA 1964) Such great extracellular action potentials were associated with long duration of the action potential in the low age groups The long duration correlated with Poissonian tendency Long duration showed a reduction to approximate adult values at the age of two weeks The shortening of the action potential indicates a change in the functional properties of the neuron membrane The associated diminution in the occurrence of giant extracellular responses could indicate a more active function of the cell membrane The associated diminution in Poissonian features of the interval sequences could reflect a more stable function of the cell This would thus be associated with functional maturation of the neuron membrane

Differences between brain regions

In many respects, two different groups of regions were distinguished, the non specific thalamic nuclei, on the one hand, and the complex, constituted by capsula interna, globus pallidus and zona incerta, on the other The differences in mean frequency between age groups were less in the non specific thalamic nuclei than in other regions Multimodal histograms were more frequent in the non specific nuclei than in the other regions

Approximately Gaussian histograms were more common in globus pallidus internal capsule and the zona incerta than elsewhere A related finding was the high incidence of periodical renewal densities in these regions The occurrence of highly regular pulse trains in these regions provides a contrast to the multimodal histograms in the non specific nuclei

The differences between regions described in terms of the principal component scores for the histogram component were similar to the differences in mean discharge rate Thus the differences between age groups were small in the non specific nuclei, and large in the other regions It has been postulated that the nuclei reticularis thalami and ventralis anterior could mediate the non specific influence to the cortex (CHOW 1952, HANBERY and JASPER 1953 HANBERY, AJMONE MARSAN and DILWORTH 1954, MAGOUN 1958) These nuclei evidenced similar functioning patterns as regards the form of the histograms and the effect of age on mean discharge rate in this study

DISCUSSION

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functions leads, according to the random walk model of GERSTEIN and MANDELBROT (1964), also to greater dispersion of intervals, which happened in the present series during puberty

Proposed models for neuronal activity in the light of this study

Random impulse sequences (Poisson processes) in the spontaneous activity may be regarded as quite exceptional in the light of this study Most of the Poisson processes were met in the young age groups Most of the histograms had a clear ascending phase, possibly explicable at times as a result of the refractory period (BISCOE and TAYLOR 1965) Even the falling phase was seldom really exponential Consequently this study provided no confirmation of the model of SMITH and SMITH (1965), based on double Poissonian showers

More often the distributions faintly resemble the gamma distribution as found by KUFFLER et al (1957) in the retinal ganglion cells, and theoretically derived for neuronal impulse activity by STEIN (1965) In most cases, however, the histograms showed definite deviations from the gamma distribution (Fig 9) No more than eight possible gamma distributions were found in 298 samples examined The random walk model of GERSTEIN and MANDELBROT (1964) is said to be defective since it does not take into account the falling phase of the individual post synaptic potentials (STEIN 1965) Nevertheless the typical histograms presented by GERSTEIN and MANDELBROT for the simulated model resemble the distributions recorded in this study The histograms showed here a distinct relationship to the mean frequency of firing, with much smaller dispersion for higher frequencies The nearly normal distributions were found only in the samples of fast mean frequency This is in agreement with the post stimulus latency histograms described by VERVEEN and DERKSEN (1965) Their model of fluctuating membrane potential at firing threshold would also describe the variation in the histograms of the spontaneous activity recorded in the current study

The possibility that the histograms could be characterized by only one parameter represented in this context by the second principal component has also been pointed out by HAGIWARA (1954) for impulse interval distributions from muscle spindle under different stimulating tensions and VERVEEN and DERKSEN (1965) for post stimulus latency histograms from frog node of Ranvier

The autocorrelations were all gathered in the first principal component and all the histogram variables in the second principal component These two combined variables seem to describe different properties of the inter

val generating process, varying independently of each other in this series. If more types of tests, not falling in any of these categories, had been applied, it is possible that more parameters would have been found. These parameters of spontaneous neuronal variability cannot yet be interpreted unambiguously.

Great variation in the nature of spontaneous impulse sequences was noticed in this investigation. The roles of different types of sequences are difficult to interpret. Some of the cells studied might have been active in some functioning circuits with unknown connections at the time of recording. As was pointed out in the introduction, according to BERGSTRÖM (1964) the suprathreshold sensory manifold of REENPÄÄ (1962) can be defined by the aid of real numbers, whereas it is possible to define the subliminal sensory quantities, related to the spontaneous activity of the corresponding sensory system, with imaginary quantities. Quantities including complex numbers could not carry typical sensory information as they have no order of magnitude. Thus the variation noted in the spontaneous impulse sequences in this study would not be associated with information transmission but might reflect the general excitability of the nervous centres examined.

SUMMARY

1. An examination has been made of the spontaneous impulse activity of 298 neural cells of 64 rabbits ranging in age from one hour to eight months. The animals were anaesthetized with urethan and local anaesthetics. The cells studied were histologically localized in the hippocampus, amygdala, globus pallidus, capsula interna, zona incerta and the thalamic nuclei reticularis, centralis lateralis, ventralis anterior, ventralis lateralis, lateralis anterior and lateralis posterior. The recording was extracellular, with glass capillary microelectrodes. The impulse intervals were measured with a general purpose computer, and were analysed by methods of statistical time series analysis: this included computation of the mean discharge rate, moments, skewness, kurtosis, histograms, autocorrelations, intensity functions and renewal densities. The results were subjected to multivariate analysis.

2. The distance between spontaneously active cells diminished slightly with age.

3. The extracellularly recorded action potential was long in duration before the age of two weeks.

4. The mean frequency of firing was low at birth, 1.5 imp./sec, but increased until the beginning of puberty, at the age of two and a half months, when it was 18.6 imp./sec. The most marked increase occurred at the age of two weeks. After puberty, the mean frequency of firing became clearly slower, being 13.4 imp./sec at the age of eight months.

5. The most usual type of histogram faintly resembled the gamma distribution, but deviated significantly from it. Exponential histograms and gamma distributions were also found. Nearly normal distributions were most frequently observed in the globus pallidus, capsula interna and zona incerta. Multimodal distributions were also found, their number increased with age. They were most frequent in the non-specific thalamic nuclei reticularis and ventralis anterior, and also in the lateralis anterior

6 The dispersion of the histogram markedly diminished with an increasing mean discharge rate The nearly normal distributions were always to be remarked in cells with a high mean discharge rate All the measurements obtained from the histogram varied jointly from cell to cell Thus, they all received high loadings in the second principal component, which could be characterized as a combined histogram variable

7 The autocorrelations with different lags often varied jointly, which was apparent in the high mutual correlations between them Thus they all received high loadings in the first principal component The values of the autocorrelations diminished slightly with age

8 Periodically oscillating intensity functions of the pulse trains were found in 18 per cent of the analysed samples Non periodic dependence between the intervals was also found In 71 per cent, the interval sequence proved to be a renewal process The frequency of periodic intensity fluctuations was not correlated with the mean impulse frequency at low discharge rates, although it was at high discharge rates The periodic intensity functions increased with age until the beginning of puberty, diminished after this, and again rose towards adulthood

9 The thalamic non specific nuclei reticularis and ventralis anterior, did not exhibit differences between age groups on their examination for the mean frequency of firing and the histogram principal component scores which were as clear as those in the other regions

10 Differences between the age groups were observed as regards the duration of the action potential, mean frequency of firing, dispersion of intervals, incidence of multimodal distributions, periodic intensity functions and renewal densities Marked changes often occurred at the age of two weeks, and at the beginning of puberty at the age of two and a half months There were postulated a change in the functional properties of the neuron membrane at the age of two weeks, and a maturation of inhibitory systems at puberty

11 The models published by others as regards the interval distributions for neuronal activity are discussed in terms of the results obtained The possibility of describing the histograms of spontaneous activity by means of a single parameter is brought to light Moreover, the study resulted in the finding of other possible parameters of the process generating the interval sequences such as the autocorrelation principal component This indicates that the spontaneous neuronal variability cannot as yet be explained unambiguously

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This summary is based on studies reported in the following papers

I Studies on the relationship between flow resistance, capillary filtration coefficient and regional blood volume in the intestine of the cat Folkow B O Lundgren, and I Wallentin Acta physiol scand 1963 *57* 270—283

II The effect of graded vasoconstrictor fibre stimulation on the intestinal resistance and capacitance vessels Folkow B, D H Lewis O Lundgren, S Mellander and I Wallentin Acta physiol scand 1964 *61* 445—457

III The effect of the sympathetic vasoconstrictor fibres on the distribution of capillary blood flow in the intestine Folkow B D H Lewis, O Lundgren S Mellander, and I Wallentin Acta physiol scand *1964 61* 458—466

IV Blood flow capillary filtration coefficients and regional blood volume responses in the intestine of the cat during stimulation of the hypothalamic defence area Cobbold, A, B Folkow O Lundgren and I Wallentin Acta physiol scand 1964 *61* 467—475

V Effects of sympathetic vasoconstrictor fibres noradrenaline and vasopressin on the intestinal vascular resistance during constant blood flow or blood pressure Dresel P and I Wallentin Acta physiol scand 1966 *66* 427—436

VI Rubidium^{86} clearance during neurogenic redistribution of intestinal blood flow *Dresel*, P, B Folkow, and I Wallentin Acta physiol scand 1966 *67* 173—184

VII Importance of tissue pressure for the fluid equilibrium between the vascular and interstitial compartments in the small intestine Wallentin, I Acta physiol scand 1966 In press

The papers are referred to by their Roman numerals in the text

General Introduction

The blood supply of the intestine serves two complex organ systems coupled in series comprising both the functional aspects of nutrition and those of the liver, which is the main site for synthetizing and regulating materials for body growth maintenance and repair Despite the paramount importance of this vascular circuit surprisingly little detail is known about the functional properties and normal regulation The relative paucity of well controlled studies may not be entirely due to neglect and lack of recognition of the problems, but rather to the complexity of the intestinal function and possibly also the relative inaccessibility of the organ system as compared to muscle or skin the circulation of which has been studied extensively Pathophysiological aspects of intestinal blood flow appears to have attracted considerable interest by the surgeon and internist who no doubt recognize the fact that dysfunction of the intestinal circulation may have a variety of serious consequences In fact intestinal infarction is usually more dangerous than infarction of the heart Thus many investigations of the intestinal circulation are concerned with shock dumping reactions obstructive ileus etc (for ref see Texter 1963, Grayson and Mendel 1965)

A large number of basic physiological questions remains to be answered How does blood flow vary with digestive activity? Is the distribution of blood flow between the intestinal tissue layers both under intrinsic and extrinsic control? Is the neuronal influence on resistance vessels capacitance vessels and precapillary sphincters of the intestine nondiscriminating or is there a selectivity of functional importance as in skeletal muscle (Mellander 1960 Cobbold *et al* 1963)? How strong is the central influence on the total intestinal blood flow and on regional distribution when performing muscle work during sudden stress or alarm reactions etc?

An endless enumeration of unanswered questions concerning the, as yet little known basic features of the intestinal vascular bed may serve little purpose except to illustrate how extremely difficult it is to attack these problems experimentally Thus technical and surgical procedures often interfere with the ability of the preparation to respond normally to physiological stimuli e g digestive activity or exercise Even though well

controlled experiments on anesthetized animals can be designed, difficulties, inherent in the complexity of the intestinal vascular bed are encountered The tissues of the intestine mucosal glands, smooth muscle fat ganglionic nerve cells various connective tissue, have all different functions and presumably different needs for nutritive blood supply In addition to the basic metabolic requirements of the mucosa, its needs for secretion and absorption require the blood supply to be regulated over a considerable range

The vascular beds of these different tissues can be considered as parallel-coupled vascular sections, with different design and capacity However, even if there are large discrepancies between flow capacities of these special tissues, their blood vessels are integrated parts of the intestinal circulation When only the total blood flow is measured, the regional flows cannot be assessed With special techniques however, utilizing isotopes and microspheres it is possible to determine the regional blood flow (cf Lindseth 1960 Rayner, MacLean, and Grim 1960, Weiner 1961, Munck *et al* 1964, Kampp and Lundgren 1966) The individual parallel coupled vascular section is built up of arterioles, capillaries and veins, *i e* vascular sections in series Instead of this morphological nomenclature the corresponding physiological terms are often used, because *resistance exchange* and *capacitance vessels* identify the functional capacity of the respective sections without commitment of structure as related to function It should be emphasized that although the two sets of terms overlap discrepancies may exist The term *resistance vessels* is used here in a manner which does not allocate the resistance to any particular morphological nor sequential section of the circulation but includes all resistances of the vascular bed Similarly the capacitance vessels corresponding mainly to the veins, include also vessels of less conspicious capacitance on the arterial side

It is not within the scope of this introduction to review all papers dealing with intestinal circulation For this the reader is referred to *e g* Bradley (1963) Grim (1963) Grayson and Mendel (1965) From studies dealing specificly with problems relevant to the present series of investigations it is known that digestive activity usually is accompanied by increased blood flow (Brodie and Vogt 1910 Brodie Cullis and Halliburton 1910 Herrick *et al* 1934 Lowenthal, Harpuder and Blatt 1952 Brandt *et al* 1955 Reininger and Sapirstein 1957 Grim and Lindseth 1958), that sympathetic vasoconstrictor activity constricts the intestinal resistance vessels (*e g* Burton Opitz 1908 Deal and Green 1956 Celander 1959 Nock 1959 for more ref see Grayson and Mendel 1965, see also discussion in chapter II) and capacitance vessels (Donegan 1921 Fleisch 1931 Bradley

et al 1953 Alexander 1954, for more ref see Alexander 1963 Bradley 1963) Precapillary sphincters of the mesentery have been observed microscopically and found to contract as a response to constrictor fibre activity (Lee 1949 Zweifach 1954)

There is little or virtually no quantitative information on the interaction between these different series coupled vascular sections In order to evaluate the functional integration of these vessels their responses were measured simultaneously The following questions related to the circulating integration of the intestinal tract were considered to be of primary interest

1 What are the relative changes in tone of the resistance vessels the capacitance and exchange vessels when blood flow varies over its maximal range? (I)

2 The intestinal mucosa was found to have a considerable capacity for transcapillary filtration as indicated by the capillary filtration coefficient (CFC) (I) What mechanisms govern the transcapillary filtration-absorption exchange and how is the intestine protected against edema formation when capillary pressure increases *e g* at increased portal pressure? (VII)

3 How do graded prolonged activations of the sympathetic vasoconstrictor fibres influence the various vascular sections? (II, III)

4 Will central activations of the sympathetic fibres *e g via* the hypothalamic defence area elicite responses similar to those produced by direct stimulation of sympathetic vasoconstrictor fibres? (IV)

5 Activations of vasoconstrictor fibres induce a specific vascular response the autoregulatory escape It seems to indicate a redistribution of blood flow from the mucosa towards the submucosa Can this response also be elicited by the infusion of vasoconstrictor agents? Will the socalled autoregulatory escape appear also when the induced stimulation is not permitted to curtail the total blood flow *i e* under constant flow condition? (V)

6 Does redistribution of blood flow involve an opening of true arteriovenous shunts or is it merely a shift in flow to submucosal capillaries as the mucosal ones constrict? (VI)

beneath the diaphragm and the peripheral ends were mounted on bipolar, silver chloride electrodes It was sometimes technically impossible to dissect free long enough sections of the right nerves to place an electrode Under such circumstances only the left ones were stimulated Supramaximal stimulation by square wave pulses (5—7 V, 2—5 msec) was delivered from a Grass stimulator (model S4E) with frequencies usually between 1—10 imp/sec occasionally 16/sec

The hypothalamic defence area was stimulated (paper IV) by bilateral steel electrodes inserted with the aid of a Horsley Clarke stereotaxic apparatus Optimal responses were obtained at 2—5 V, pulse duration of 2—5 msec and frequencies of 50—90/sec

Isotope experiments Changes in regional blood volume were distinguished from volume changes caused by transcapillary fluid shifts through tagging of red cells with Cr^{51} (Owen 1959) and recording the γ activity from the preparation in the plethysmograph (VII)

To investigate the possibility of shunting during sympathetic stimulation the arterio venous extraction of $rubidium^{86}$ was recorded (paper VI) using either single injection or constant infusion techniques The capillary transport coefficient PS was calculated (Renkin 1959 Renkin and Rosell 1962 a) PS is expressed in ml/(min x 100 g tissue) and corresponds to the maximal clearance at infinite blood flow and prevailing capillary surface area and permeability For basic premises and interpretation see VI PS may also be considered a measure of capillary exchange area available for diffusion as is CFC for filtration

Determination of the capillary filtration coefficient, CFC

The determination of CFC, as a measure of capillary exchange area is based on the assumption that in capillaries closed at the arterial end by their precapillary sphincters negligible fluid shifts will occur between the stagnant capillary content and the interstitial fluid also when hydrostatic pressure differences are induced from the venous side A new equilibrium will soon be reached and the volume transfer involved must be insignificant in comparison with the amount of filtration and absorption that occurs in capillaries open to blood flow If this were not true, clearcut differences would not be seen between CFCs measured at various degrees of vascular tone, graded vasoconstrictor fibre stimulation, etc

To determine CFC the venous pressure is suddenly elevated by a known amount usually 10 cm H_2O A characteristic biphasic volume response occurs initially the intestinal volume increases rapidly usually within

10—15 sec Concurring with this sudden volume change a longlasting slow increase takes place As interpreted by Lewis and Grant (1925) the two phases are due to venous distension and outward filtration, respectively Further experimental work has confirmed this interpretation and demonstrated that the slope of the slow phase can be used as a quantitative measure of the net capillary filtration (Pappenheimer and Soto-Rivera 1948 Mellander 1960 Johnson and Hanson 1962 1963) From this slope and the increase in mean capillary pressure the CFC may be calculated and expressed as the amount of filtrate produced per minute in 100 g of tissue per mm pressure difference induced across the capillary membrane (ml/(min × 100 g × mm Hg)) Problems involved in determinating the slope and the change of mean capillary pressure need some remarks

When the Starling equilibrium is upset by a rise in capillary pressure fluid will be filtered from the capillaries open to blood flow This filtered fluid will increase the hydrostatic pressure outside the capillaries i e the tissue pressure and will also dilute the interstitial proteins, thereby lowering the colloid osmotic pressure of the interstitial fluid In turn the effective transcapillary pressure gradient will decrease and hence the outward filtration also gradually decreases Therefore the recorded slope will on the one hand tend to underestimate the true CFC depending upon the extent to which the mentioned factors of the Starling equilibrium build up as counterforces It is on the other hand, known that veins display some delayed dilation when they are distended (Alexander, Edwards and Ankeney 1953 Alexander 1963 Johnson and Hanson 1963) Therefore if the slope is based on a part of the volume curve where delayed venous filling takes part the filtration slope will be overestimated

The relative importance of these factors was studied in paper VII It was found that delayed relaxation of the veins undoubtedly occurs, but may be unimportant except during the first 40—50 sec after an acute elevation of the venous pressure (cf John on and Hanson 1963) In fact, the greater part occurs between 0—30 sec and none of our estimations of CFC have been based on observations during this period Some of the values in paper I appear to overestimate CFC due to such a delayed venous filling This overestimate is maximally of the order of 15—20 per cent if the conclusions from paper VII are correct The CFC estimates in paper I may be lower than the true CFC of the intestine as the preparation included lymph glands up to 30—40 per cent by weight and because the CFC of lymph glands has been shown to be considerably lower (Lundgren and Wallentin 1964) The error introduced by not correcting for the lower CFC of the lymph glands is rather insignificant in paper II—VII, since a

larger plethysmograph was used, permitting utilization of a relatively much larger section of the intestine In these experiments lymph glands only constituted 5—10 per cent of the total weight In retrospect the two mentioned opposing errors almost cancel each other in our first study where absolute values are of importance

In paper VII it was shown that following elevation of venous pressure outward filtration gradually decreased and sometimes a new isovolumetric state was reached within 10—15 min All slopes utilized for the determination of CFC were carried out during the period 0 5—2 0 min after venous pressure elevation The compensatory effects counteracting filtration, i e increasing tissue pressure and concomitant lowering of the tissue colloid osmotic pressure, do not appear to have been of appreciable magnitude during this period and may only have lowered the estimates of CFC with less than 10—20 per cent

On the one hand, it has been pointed out possible errors in the estimation of CFC, on the other hand, it is equally important to stress that none of these possible errors in the determination of CFC have significant influence on the evaluation of *relative* changes in CFC, such as they occur in connection with vasoconstriction and vasodilation The displacement also of the curve in Fig 4 of paper I, up or down within reasons, is of no consequence for the main conclusion drawn *viz* the capillary surface area of the small intestine is very large in comparison with skeletal muscle

To evaluate the change of mean capillary pressure, produced by raising venous pressure it is necessary to know the ratio between the pre and postcapillary resistances (Bayliss and Starling 1894, Pappenheimer and Soto Rivera 1948) It is necessary to know the mean capillary pressure at steady state for calculation of this ratio Because of the high concentration of the proteins in the intestinal lymph, indicating a fairly high colloid osmotic pressure of the interstitial fluid it was considered likely that the capillary pressure in the intestine is somewhat lower than in e g skeletal muscle Therefore 15 mm Hg was chosen as a reasonable mean value for the intestinal capillary pressure in a complete Starling equilibrium This would mean that 85 per cent of an increment in venous pressure would be transmitted to the intestinal capillaries when the arterial pressure is 100 mm Hg and the venous pressure close to zero This value 85 per cent, has been used in the calculation of CFC throughout all the present papers

Both seemingly high and quite low capillary pressures have been reported for the intestine Measuring capillary pressure with the direct micropipette technique of Landis (1926) Koniges and Ottó (1937) found 26 8 and 31 3 mm Hg for the venous and arterial end respectively Johnson (1965) in

contrast, in experiments utilizing his isogravimetric stop-flow technique, at venous pressure close to zero arrived at an exceedingly low mean value of 9 2 mm Hg for the intestinal capillary pressure

It is somewhat difficult to assess the reasons for this rather large discrepancy between the reported values Vasodilation in general, irrespectively cause, is known to increase the capillary pressure Koniges and Otto (1937) used a single villus preparation It is possible that this technique involves extensive preparation and handling of the tissue and that venous stasis with correspondingly higher capillary pressure might ensue From their report it is also not quite clear whether transcapillary equilibrium existed during the observations Johnson in his report despite the low mean capillary pressure suggested that only 62 per cent of a venous pressure increment is transmitted to the capillary level mainly because of increased myogenic precapillary activity in response to the pressure increase This myogenic activity would secondarily tend to lower the capillary pressure If this is generally true the CFCs measured in our studies would actually be about 25 per cent larger than those values reported

There are certain basic assumptions in Johnson s stop-flow technique that are difficult to accept Thus, in capillaries without flow a new Starling equilibrium will be reached almost instantaneously by relatively small fluid shifts following a change in hydrostatic pressure As mentioned this is a prerequisite for the determination of CFC Judged from the weight record in Johnson s study (Johnson 1965 Fig 1) the intestinal weight decreased significantly following arrest of flow Attempts to evaluate the capillary pressure were not made until the weight had decreased to a new level It is therefore difficult to evaluate whether or not the Starling equilibrium remains unchanged This is important because the method is based on the assumption that the Starling equilibrium is maintained Furthermore stopping the blood flow, even for those few minutes necessary for stop flow determinations provokes gradual changes in vascular tone involving also the capacitance section Even if no filtration or absorption occurs at the capillary level it would seem possible to balance this changing capacitance by means of a venous reservoir and obtain isogravimetric conditions In other words it is difficult to see how Johnson s stop flow method can evaluate the intestinal capillary pressure

Even if all the mentioned objections to the stop-flow method were invalid 62 per cent as proposed by Johnson may not be used for calculation of the CFC values in paper I because isopropyl noradrenaline was given to increase the blood flow, and this substantially decreases the precapillary myogenic responses to elevated pressure Regardless of the argu

mentation above one cannot exclude the possibility that a value for the ratio different from that currently used (85/15) might have been a better approximation, in particular perhaps in those cases where no vasodilator substances were given, e g paper II—IV, VI Nevertheless, as pointed out previously, even relatively large changes of this ratio influence the CFC only slightly (see discussion in I) and the conclusions about the relative changes of CFC would not be seriously affected

Results and Discussion

I Quantitative aspects of the intestinal circulation

The maximal range of some important functions of the intestinal circulation was studied in paper I and VII

Resistance vessels Following acute denervation of the intestinal vascular bed the resistance vessels exhibited a considerable basal tone Usually, the rate of blood flow of denervated resting intestine was in the range of 40—60 ml/(min x 100 g intestine) but values outside this range, especially very high ones were also observed When maximal vasodilation was induced by isopropyl noradrenaline, flows up to 275 ml/(min x 100 g x 100 mm Hg) were recorded

As it appears to be generally agreed that vasodilator fibres to the intestine do not exist (Bayliss 1923 Bulbring and Burn 1936 Gernandt and Zotterman 1946 Folkow, Frost and Uvnas 1948 Celander and Folkow 1951, Kewenter 1965), local metabolic control must span over a considerable range and be capable of providing sizable blood flow reserve It is reasonable to assume that the blood flow of the smooth muscle part of the intestine does not exceed that of the skeletal muscle considering the relatively low metabolic demands of smooth muscles in general (cf Munck *et al* 1964 Kampp and Lundgren 1966) The mucosa-submucosa which to a considerable extent consists of glandular tissues must therefore have a maximal blood flow capacity of about 500 ml/(min x 100 g) similar to that of the salivary glands (Terroux Sekelj and Burgen 1959 Martinson and Odelram 1966) This huge blood flow capacity is presumably a prerequisite for the supply of sufficient raw materials for secretion at maximal digestive activity and for transport of absorption

Noradrenaline (I) or stimulation of the vasoconstrictor fibres (II) could decrease the intestinal blood flow to a few ml/min, but only temporarily This will be discussed in more detail in next chapters

A wide range of values for resting blood flow can be found in the literature (for ref see Grim 1963 Grayson and Mendel 1965) It should be noted that resting gastrointestinal blood flow is a rather tenous concept particularily because the degree of prevailing activity is difficult to ascertain The term is used in the present papers (I—VII) for practical

outward filtration from the blood stream 3 Dilution of the interstitial proteins gradually lowering the colloid osmotic pressure of the interstitial fluid Ad 1 Myogenic responses presumably are of considerable importance in preventing edema in intact animals They were, however largely eliminated in the current experiments where the gradual decline of filtration was studied by two different methods Firstly, by always infusing isopropyl noradrenaline or papaverin *i a* which dilates the vessels and in larger concentrations eliminates their reactivity Secondly, by measuring the differences in rate of filtration or absorption between two equal separate elevations of the venous pressure before and after a period of further filtration caused by still higher venous pressure It could here be expected that equal elevations of venous pressure would cause the same myogenic response, if any If such a myogenic response were the main factor reducing the filtration the same change of filtration would have occurred Under these experimental circumstances great differences in filtration rate were observed, usually, an outward filtration during the first period of venous pressure rise was reversed to absorption during the second period When these differences in filtration rate were divided by the simultaneously measured CFC, the corresponding displacement of the Starling equilibrium is obtained The mean shift in the transcapillary balance during this procedure in 44 determinations amounted to $9\ 1 \pm 2\ 33$ (SD) mm Hg and values up to 15 mm Hg were recorded As special precautions were taken to exclude myogenic responses an increased tissue pressure and/or a decreased interstitial colloid osmotic pressure are likely to have been the only factors which reasonably could account for this displacement of the Starling equilibrium

Recently, Johnson and Hanson (1963) reported that dog intestine could reach a new isogravimetric state for venous pressure levels up to 18 mm Hg Since the tissue pressure of the mucosa did not rise as measured by a needle technique it was concluded that the ensuing dilution of the interstitial proteins was responsible for the new Starling equilibrium However analoguous interpretation would for the present investigation infer a mean value of the interstitial colloid osmotic pressure of the intestine of up to 15 mm Hg which is in the range of the colloid osmotic pressure of the intestinal lymph in the cat (Morris 1956) There are good reasons to assume however that the mean colloid osmotic pressure of the lymph is far greater than that of the interstitial fluid of the same tissue (Landis and Pappenheimer 1963) Conversely the mean interstitial colloid osmotic pressure of the intestine must be well below this figure Moreover, it was calculated that the amounts of fluid filtered were so small that if they should account

for the whole displacement of the transcapillary equilibrium by the process of protein dilution the interstitial fluid compartment must amount to only 2—4 per cent of intestinal weight. Actually measured values of the interstitial space of the intestinal tissue have been reported to be from 20—40 per cent depending on method used (Bozler 1961 Goodford and Hermansen 1961 Davenport and Alzamora 1962 Barr and Malvin 1965) Therefore neither of these two possibilities was considered plausible For these reasons dilution of the interstitial proteins was thought to be quantitatively less important than the increasing tissue pressure in counteracting outward filtration Most probably both factors are involved Possibly the reason why Johnson and Hansson (1963) did not record any increasing tissue pressure with their needle technique is that the pressure is built up in tissue spaces which are very much smaller than their smallest needle (McMaster 1946 Guyton 1963) *A priori* it is unlikely that any tissue *should so entirely lack cohesive forces that it would allow intercellular* fluid accumulation without any change in tissue pressure

In their classic study Landis and Gibbon (1933) used a technique very similar to the one used in the present investigation They concluded that outward filtration increased the tissue pressure in human forearm They also measured the differences in the rate of filtration during periods of raised venous pressure, observing that changes of direction of transcapillary fluid movement could occur if only enough fluid was filtered in the meantime

II Nervous control of intestinal vessels

Intestinal circulation was studied (II—IV) during vasoconstrictor fibre stimulation, either directly through the splanchnic nerves or *via* the hypothalamic defence area Continuous graded activation of the sympathetic vasoconstrictor fibres produced a characteristic vascular response Initially, the blood flow decreased in proportion to the stimulation frequency used Occasionally high 'physiological' frequences, 8—10 imp/sec, produced complete cessation of flow during the initial 20—40 sec However, despite a continued stimulation, the rate of blood flow increased again within 2—3 min, often reaching the prestimulatory level or sometimes even higher flow rates Usually, however, flow stabilized at levels of approximately 20—30 per cent below control This ability of the intestinal vascular bed to maintain flow in the face of sympathetic stimulation was termed 'autoregulatory escape', because it is a local response Changed activity of the sympathetic system is obviously not the cause inasmuch as the neuronally induced vasoconstriction of the capacitance vessels and precapillary sphincters was maintained during the 'escape' The possible failure of transmitter release was ruled out in another study (V) *(vide infra)* The rate of blood flow during the steady state phase of the 'autoregulatory escape' was usually lesser at low stimulation frequencies (2—6/sec) than at high ones (8—10/sec) In the latter case blood flow during established escape was often the same as the prestimulatory flow (II, Fig 2)

Despite the maintenance of blood flow within control range, a reactive hyperemia was seen almost regularly after interruption of the sympathetic stimulation Such a hyperemia was seen also in cases where the 'steady state' level of blood flow had been *greater* than the control flow, a situation in which reactive hyperemia would not be expected to occur In a few cases 'autoregulatory escape' was not encountered and then no reactive hyperemia occurred either in spite of the sustained vasoconstriction and decreased blood flow during the period of stimulation

The rapid change of the intestinal volume during stimulation is assumed to be due to rapid changes in regional blood content, *i e* expressing the degree of filling of the capacitance vessels The response of the capacitance vessels to the stimulation was as prompt as those of the resistance vessels, and suggests an almost instantaneous reduction of up to 40 per cent in

regional blood content The degree of the initial emptying of the capacitance vessels was as for the resistance vessels proportional to the frequency of stimulation reaching a maximum at 8—10 imp/sec This initial capacitance response was interpreted as due to two different mechanisms 1 active vasoconstriction of the veins and 2 resilient forces of the vessel wall and final collapse of the vessels because of the lowered hydrostatic pressure

The relative importance of these two mechanisms, one 'active and one passive depends on venous outflow pressure On the one hand at low venous pressure the mean distending pressure of the veins will decrease when precapillary constriction lowers the transmural pressure at the capillary level This results in a passive emptying of the venous capacitance section High levels of venous outflow pressure on the other hand is associated with high mean transmural pressure in the venous compartment, and this pressure will be only little reduced by a precapillary vasoconstriction Hence the veins will remain distended and in this situation venous emptying is mainly due to active vasoconstriction (Mellander 1960 Öberg 1964) For these reasons venous emptying is more dominated by active venoconstriction at high venous pressures and by forces inherent in the vessel wall and parenchymal tissues at low levels of venous pressure

When passive emptying had occurred during the *initial* vasoconstriction the volume also increased as the blood flow increased A considerable reduction in blood volume was apparent throughout the steady state of the stimulation period however as compared with the control level These volume changes secondary to the autoregulatory increase of flow were not seen if the venous pressure was as high as 10—15 cm H O i e high enough to prevent passive emptying during the constriction The degree of intestinal blood volume reduction maintained during stimulation was interpreted as due to active vasoconstriction of the veins now revealed when the capillary pressure was restored again The maximum of this active constriction was reached already at 4—6 imp/sec similar to the responses of the capacitance vessels of skeletal muscle (Mellander 1960)

When the autoregulatory escape was completed i e during the steady state' phase of flow the volume was as a rule not changing either indicating a transcapillary equilibrium with no filtration or absorption As this equilibrium was quickly reached the only reasonable interpretation is that the mean capillary pressure is the same as during the isovolumetric state before stimulation This type of response with respect to the pre-postcapillary resistance ratio is quite different from that seen when stimulating sympathetic fibres of skin or of skeletal muscle where a maintained

decrease in mean capillary pressure causes a continuous absorption of interstitial fluid, (Mellander 1960, Öberg 1964) an important compensatory fluid shift following hemorrhage

During the steady state phase of sympathetic stimulation the capillary surface area, as determined by CFC, was usually reduced to 30—50 per cent of the prestimulatory value Unfortunately it was not feasible to obtain an estimate of CFC during the initial vasoconstriction India ink injected during this phase indicated however, that the mucosa suffered extensive ischemia *i e* CFC would probably be close to zero (see IV, Fig 3 B)

After interruption of the sympathetic stimulation an overshoot of both volume and CFC was seen during the phase of reactive hyperemia

Evidently, the capillary surface area available for filtration was decreased out of proportion to the decrease of blood flow during the autoregulatory escape This could mean that the blood is passing mainly through preferential channels' as described in the mesentery by Chambers and Zweifach (1944) The preponderance of such preferential channels in the intestine has never been established (see Baez 1959, Wiedeman 1963) An alternative interpretation would be based on abundant arteriovenous anastomoses in the submucosa described by Spanner (1932) That at least part of the blood was passing through such submucosal connections during nerve stimulation was corroborated by the results of intra arterial injections of India ink during the different phases of the autoregulatory escape (III, IV)

The reactive hyperemia following the stimulatory period indicates that at least some part of the intestine has been deprived of blood flow This observation clearly indicates that despite a prominent escape of some resistance vessels re establishing over all blood flow, selective neurogenic mechanisms are in operation to redistribute blood flow A further support of this concept is gained by the simultaneous reduction of CFC A decrease of CFC to 40 per cent without concurrent reduction in blood flow must mean that the blood passes through the capillary bed with a linear flow rate more than twice that of control, *i e* uneven distribution of capillary flow (physiological shunting), or that some 60 per cent of the blood is passing through true arterio venous shunts Other plausible interpretations of the data are possible such as intermittent vasodilation (localized reactive hyperemia) thus accommodating a greater volume flow with a simultaneous reduction in available filtration area It was shown in paper VI that the autoregulatory escape does not involve an opening of true arterio venous shunts Therefore an uneven distribution of capillary blood flow,

irrespective mechanism, would appear to be secondary to the primary neurogenic constriction, manifesting itself as the socalled autoregulatory escape

It is possible that a greater part of the capillaries are bypassed during the manifest escape because their precapillary sphincters are closed Another possibility is that the stimulation primarily affects arterioles and larger vessels Moreover the combined involvement of all the suggested mechanisms cannot be excluded It is noteworthy however, that the mucosal capillaries were closed and that the ischemic features predominated throughout the stimulatory period The nervous control of the capillary blood distribution in the intestine is therefore more pronounced than in the resting skeletal muscle where local metabolic factors easily override neurogenic influence In contrast to the behaviour of the intestine, blood flow of skeletal muscle is considerably reduced throughout the period of sympathetic stimulation and the initially lowered CFC rapidly reverts to control levels or higher (Cobbold *et al* 1963) Despite a substantial reduction in blood flow it is conceivable that a larger number of true capillaries open and increase the capillary exchange area

When India ink was injected during the steady state phase of stimulation less ink particles were seen in the mucosa and in the muscular part of the intestine as compared to the submucosa It was therefore concluded that the blood flow is shifted to a considerable extent towards the submucosa This should not be construed to mean that the entire autoregulatory escape phenomenon is due to such a shift A secondary increase must occur also in the mucosa inasmuch as more India ink was found during the steady state phase of vasoconstrictor fibre stimulation than that found during the initial maximal constriction

In paper IV the sympathetic vasoconstrictor fibres were activated by topical stimulation in the hypothalamic defence area (Eliasson *et al* 1951 Abrahams Hilton and Zbrozyna 1960 1964) The responses of the intestinal vascular bed were identical with those obtained by direct stimulations of the vasoconstrictor fibres

Several investigators have studied the influence of splanchnic nerve stimulation on the intestinal circulation since the concept of vasoconstrictor fibre control of the vascular bed was introduced by Claude Bernard (for ref see Bunch 1899 Grayson and Mendel 1965) It seems therefore strange that nobody has described the autoregulatory escape before Earlier investigators using plethysmographic techniques have interpreted volume decreases as decreases of blood flow instead of reduction of blood content (e g Hallion and Francois Franck 1896 Bayliss and Starling 1899 Bunch

1899). Moreover, the periods of stimulation have often been too short to allow the escape phenomenon to appear (e.g. Burton Opitz 1908, Kock 1959).

Deal and Green (1956) stimulated the splanchnic nerves in the dog but did not report the duration of stimulation. They used 'supraphysiological' frequencies, 20 imp/sec, which cause a different type of escape phenomenon in most vascular beds (Celander 1954) presumably related to a gradual depletion of transmitter. They reported that blood flow increased during the stimulation period without giving details about the magnitude. Celander (1959) observed, following an initial blood flow decrease induced by vasoconstrictor fibre stimulation in the intestine of the cat, that blood flow increased again and interpreted this escape from vasoconstrictor influence as due to metabolic factors.

It is of considerable interest that Öberg (1964) confirmed the present results fully, utilizing induced reflex increases in tonic vasoconstrictor activity in different ways, e.g. by graded bleeding, carotid occlusion and chemoreceptor activation.

Although all of our experiments have been carried out on cats, indirect evidence suggests that similar vascular adjustments occur also in other species, e.g. in dog or man. Thus in the dog little or no increase in mesenteric resistance is usually seen following hemorrhage (Heinemann, Smythe and Marks 1953, Reynell *et al.* 1955, Sapirstein, Buckley, and Ogden 1955, Cull, Scibetta and Selkurt 1956, Selkurt and Brecher 1956, Levy 1958, Smythe 1959, Friedman 1961). It has also been observed that during hemorrhage regional flow of the splanchnic area constitutes a relatively larger fraction of cardiac output than normal (Blalock and Levy 1937, Reynell *et al.* 1955, Frank *et al.* 1956, for further ref. and discussion of this problem see Grayson and Mendel 1965). Similar observations have also been made during total body perfusion at low flow rates simulating bleedings (see Andersen *et al.* 1961, Johnson, Gott and Welland 1961).

Furthermore, Bradley and co-workers (see e.g. Reynell *et al.* 1955, Bradley *et al.* 1953, Bradley 1955, 1958, 1963) have reported repeatedly that bleeding in both man and dog has no or only minor effect on the splanchnic vascular resistance but that the splanchnic blood volume decreases, often to some 60—70 per cent of control volume. These studies, performed on intact dog or on unanesthetized man, indicate that conclusions based on the present studies of the cat intestine may have a more general significance.

The effect of nerve stimulation on the intestinal CFC has not been reported before. However, Zweifach (1954) observed that the precapillary

sphincters of the mesentery were more sensitive to nerve stimulation than other vascular structures These strategically located smooth muscle cells also reacted to lower concentrations of noradrenaline when applied topically (see also Chambers and Zweifach 1944) Utilizing direct microscopic observations Lee (1949) studied guinea pig mesentery prepared during local anesthesia Provoked alarm reactions caused the precapillary sphincters to close and caused larger arterioles to narrow at some key points producing complete arrest of blood flow in capillaries arterioles and even in small arteries In spite of this a continuous blood flow was seen in larger venules and veins These direct observations are in general agreement with the present findings

Many workers have tried to assess the influence of the sympathetic system by comparing the intestinal blood flow before and after denervation (see Grayson and Mendel 1965) However the existance of the autoregulatory escape phenomenon makes such determinations relatively fruitless Volume changes resulting from denervations of the capacitance vessels would perhaps reveal more about the vasoconstrictor tone in different situations

Thus the conventional concept that the splanchnic area plays a major role in the redistribution of blood flow, *i e* that considerable reduction of its blood supply occurs during shock in alarm situations etc must be reconsidered No doubt substantial changes in this direction occur initially but the onset of the autoregulatory escape reverses this adjustment The important feature of the vascular adjustment is however that a considerable autotransfusion may be achieved by the sustained constriction of the intestinal capacitance vessels When the mucosa is deprived of blood flow there is a further economizing with available volume through curtailment of secretion The simultaneous inhibition of peristaltic movements of the intestine by adrenal catecholamines (Kock 1959) and also by sympathetic inhibitory fibres on the intramural ganglionic plexus (Norberg 1964 Kewenter 1965) reduces the need for oxygenation and blood flow Possibly the fact that the vasoconstrictor fibre activity does not depress intestinal blood flow more drastically may be of great importance for the liver in situations of emergency and exercise If the metabolic demands of the gastrointestinal tract are reduced as suggested the portal blood supply might during intestinal vasoconstrictor fibre activity provide the liver with even *more* oxygen than it otherwise would receive

III: Some characteristics of the autoregulatory escape phenomenon

Some particular aspects of the autoregulatory escape were studied in paper V. The possibility of the escape being causally related to local accumulation of metabolites overriding neuronal control was studied in this series of experiments under conditions of constant blood flow. Under these conditions the resistance vessels exhibited an autoregulatory escape during stimulation similar to that seen under conditions of perfusion at constant pressure. The resistance changes have the same time course, i.e. an initial constriction, an escape and a poststimulatory reactive hyperemia. These observations support the hypothesis that the redistribution of blood flow is strongly affected by local mechanisms overriding sympathetic vasoconstrictor influence. The existance of both the escape phenomenon and the reactive hyperemia is equally strong evidence that some part of the intestinal tissue becomes ischemic and remains so during constrictor fibre stimulation also when the overall blood flow is maintained.

On the basis of the present observations specific neuronal mechanisms cannot be excluded as participating in the redistribution of flow. In this regard it is of interest that recent studies utilizing histochemical techniques appear to suggest a relatively greater density of adrenergic fibres ramifying the small arteries of basal mucosa (Norberg, personal communication).

Noradrenaline was infused intra arterially both during constant pressure and during constant flow perfusion. In such experiments the catecholamine is distributed homogeneously to the entire vascular bed in contrast to the more restricted distribution when released from nerve endings.

In these experiments the intestinal resistance vessels also showed the typical autoregulatory escape. The escape was, however, usually somewhat more sluggish and not as complete as that during nerve stimulation. The fact that also infused noradrenaline evokes essentially the same response as neuronal stimulation suggests a characteristic distribution of α receptors in the intestinal vascular bed (Ahlquist 1948, Green and Kepchar 1959) corresponding to the distribution of sympathetic nerve endings. Cf. the irresponsiveness to noradrenaline of the umbilical artery, which has no sympathetic vasoconstrictor fibres and normal responses to other constrictor agents, e.g. serotonine (Davignon, Lorenz and Shepherd 1965). If the distribution of α receptors were uniform in all vascular resistance

sections, one would expect a sustained vasoconstrictor response to noradrenaline in particular during constant flow perfusion Actually vasopressin a potent intestinal vasoconstrictor (Bainbridge and Trevan 1917 Clark 1928, McMichael 1932, Katz and Rodbard 1939 Shaldon *et al* 1961 Haddy *et al* 1962, Texter *et al* 1964 Tsakiris Haemmerli and Buhlmann 1964) caused sustained vasoconstriction during a period of infusion and reactive hyperemia did not appear after interruption of the infusion Thus, the autoregulatory escape phenomenon is not a general response of the intestinal resistance vessels to all vasoconstrictor agents

It was frequently observed that the mere cannulation of the superior mesenteric artery, and even more so the use of perfusion pumps (Sigma motor Harvard) influence the autoregulatory escape Intestinal vascular reactivity was much depressed and the escape during vasoconstrictor fibre stimulation was sluggish and reduced sometimes even abolished (see V, Fig 2) Also autoregulation of blood flow in response to blood pressure variations deteriorated when mechanical pumps were used Although it is possible that these phenomena are unrelated there can be no doubt that both depend on an impaired vascular reactivity (see Folkow 1952 Chambers and Zweifach 1944 Johnson 1964) It may be assumed therefore that the use of mechanical pumps somehow induces changes that impair the normal reactive responses of the intestinal vascular bed to pressure changes and to vasoconstrictor fibre stimulation (see Selkurt Scibetta and Cull 1958 Hinshaw 1962)

Summary and Conclusions

The intestinal vascular bed was studied during a maximal range of induced changes in vascular tone produced either by pharmacological agents or by stimulation of sympathetic vasoconstrictor fibres. By combining a direct recording of blood flow and tissue volume the individual behaviour of the pre- and postcapillary resistance vessels, the capacitance vessels and the precapillary sphincters were studied. Estimates of the capillary filtration coefficient (CFC) were used to determine and evaluate changes of the proportion of capillaries actively circulating blood. Changes in mean capillary pressure, produced by shifts in the pre- to postcapillary resistance ratio could be estimated from changes in volume and CFC. Tracer techniques were used for investigation of some aspects of regional blood volume shifts and the existence of shunting. From the data obtained in these investigations the following conclusions may be drawn.

1. Resting blood flow in denervated small intestine of the cat is usually in the range of 40—60 ml/(min x 100 g) and the blood flow may be increased experimentally up to about 275 ml at a pressure head of 100 mm Hg. Blood flow covaries with CFC when a graded vasodilation is induced. It is about 0.10—0.15 ml/(min x 100 g) at rest and about 0.3—0.5 ml during maximal vasodilation. Assuming a maximal blood flow and CFC of intestinal muscle layers commensurate with values of other muscle tissues, the maximal blood flow of the mucosa can be calculated to amount to 500 ml/(min x 100 g) and the maximal CFC to approximately 1.0 ml (min x mm Hg x 100 g), values in the range of maximal flow in salivary glands and a filtration capacity of human kidneys respectively.

2. When prolonged elevation of the mean capillary pressure is induced from the venous side the filtration will gradually decrease and sometimes cease within 10—15 min. It was concluded that increasing tissue pressure in addition to dilution of interstitial proteins is an important mechanism for the protection against edema.

3. Prolonged stimulation of the intestinal vasoconstrictor fibres induces a specific circulatory pattern termed autoregulatory escape. It is characterized by an initially pronounced constriction of the vascular bed followed by an escape of the resistance vessels from the nervous influence usually within 1—2 min, while the perfused capillary exchange area remains reduced and the capacitance vessels maintain their constriction.

Therefore, after the initial phase of elevated resistance a sustained increase in activity of the vasoconstrictor system is revealed only by a decreased capacitance and a lowered CFC These apparently paradoxical results indicate a shift or an autotransfusion of blood from the intestine to the general circulation without corresponding increase in resistance

4 When steady state is reached during constrictor fibre stimulation, the mean capillary pressure is normalized and usually maintained at the prestimulatory level Therefore the pre postcapillary resistance ratio, which increases during the initial phase of vasoconstriction is also restored to the prestimulatory level

5 During the first 1—2 min of the poststimulatory period the whole vascular bed dilates to a degree greater than that prior to stimulation Thus reactive hyperemia is observed even when no decrease in blood flow occurs during the steady state phase of constrictor fibre stimulation

6 These characteristic changes in flow pattern induced by sympathetic activity are not qualitatively different when the blood flow is kept constant The flow pattern is also mimicked when the sympathetic transmitter agent noradrenaline is infused intra arterially during either constant pressure or constant flow However, the characteristic changes are not induced by all vasoconstrictor agents, thus vasopressin causes a sustained constriction of the resistance vessels which is not followed by reactive hyperemia

7 The autoregulatory escape phenomenon occurs also when the sympathetic system is activated by stimulations of the hypothalamic defence area

8 The autoregulatory escape may be considered a redistribution of blood flow to some extent from the mucosa towards the submucosa, because *a* A reduction of capillary exchange area to 30—50 per cent of the prestimulatory level without corresponding increase in resistance must itself indicate a redistribution of blood flow *b* The occurrance of reactive hyperemia when there is no curtailment of blood flow during constrictor fibre stimulation would appear to indicate a regional decrease in blood flow of some areas and therefore an increase in that of others *c* India ink injections intra arterially before during and after stimulation show that less ink particles reach the mucosa during the steady state than before stimulation

9 The regional increase in blood flow during the steady state phase of vaso constrictor fibre stimulation is not due to opening of true arterio-venous shunts If preferential channels or other low resistance vessels are included in the response pattern they must allow for diffusion exchange of solutes

and can therefore not be considered true shunts The discrepancy between blood flow and the perfused capillary surface area during constrictor fibre stimulation appears to be essentially a matter of uneven perfusion *i e* physiological shunting

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THE CHEWING APPARATUS

An Electromyographic Study of the Action of the Muscles of Mastication and its Correlation to Facial Morphology

BY

EIGILD MØLLER

COPENHAGEN
1966

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Eigild Møller

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Chapter I

INTRODUCTION

ABSTRACT

The study presented in this report deals with (1) the methodology of electromyographic recordings from the muscles of mastication (2) the activity in these muscles during chewing and swallowing at rest and during full effort in young adult males and (3) the correlation between the activity in these muscles and the morphology of the cranial base the jaws and the alveolar and dental arches In the introduction the physiological basis of electromyography is reviewed with special reference to its application for the study of muscle coordination

Methodological study

To reduce the influence of the recording electrodes on function it was desirable to use surface recording as far as possible A comparison of unipolar and bipolar surface electrodes showed that conducted activity from adjacent muscles was picked up by the unipolar technique In bipolar recording with an interelectrode distance which is small as compared to the size of the muscle conducted activity from other muscles is nearly identical on the two leads and therefore rejected In the bipolar surface recording the mean voltage varied with the distance between the electrodes their position on the muscle and their size In previous measurements of resistance between surface electrodes the capacitive component had been disregarded This component is important in the frequency range of surface electromyograms The impedance of surface electrodes varied widely depending on individual differences between the electrodes on their location and on the preceding treatment of the skin

A comparison was made between recording with an electroencephalograph with inkwriters and an electromyograph with a high input impedance and photographic recording from oscilloscopes Due to its low input impedance and low upper frequency limit the electroencephalograph caused a reduction

Muscle activity during swallowing

In contrast to chewing swallowing was characterized by a synergistic activation of all the muscles under study The activity in the lower lip initiated swallowing and was followed shortly by the internal and external pterygoid and the mylohyoid muscles The onset of activity was synchronous in the temporal and digastric muscles but strong activity in the digastric was delayed 150 msec relative to the temporal The upper lip was innervated in time with the temporal muscle the masseter muscle slightly later The degree of activity in (1) the anterior and posterior temporal and the masseter (2) the mylohyoid and the digastric and (3) the orbicularis oris muscles varied during swallowing in the same way there was no association between the innervation of the three groups of muscles

Tooth contact during swallowing occurred constantly in 23 of 36 subjects 7 subjects swallowed without tooth contact and in 6 contact occurred irregularly Subjects with tooth contact during swallowing had stronger activity in the anterior temporal the masseter and the orbicularis oris muscles than subjects without contact

Postural activity

With the mandible at rest the activity was slight (2–5 per cent) as compared to that observed during chewing and swallowing Elevation of the mandible from the position at rest to the position of earliest tooth contact was accomplished by a slight increase of the activity in the temporal and external pterygoid muscles

Full effort

The degree of activity in the elevator muscles during maximal bite in the intercuspal position represented the highest degree of activity in 30 of 36 subjects

Muscle activity and facial morphology

A curved cranial base was associated with strong activity in the anterior and posterior temporal and in the masseter muscles during swallowing A curved mandibular base and prognathism and anterior inclination of the mandible was associated with (a) strong activity in the anterior temporal and the masseter muscles during maximal bite in the intercuspal position and (b) a pro-

longed initial phase of low activity in the mylohyoid muscles during swallowing of saliva A large overbite was associated with (a) strong activity in the posterior temporal muscles during chewing and during maximal bite in the intercuspal position and (b) early activation of the external pterygoid and the digastric muscles during chewing

The activity in the lips during swallowing depended both on the degree of lip insufficiency (morphology) and on the time course of the activity in the mylohyoid muscle

REVIEW OF THE LITERATURE

The action of the muscles of mastication during chewing and swallowing cannot be measured directly The electrical activity of the muscle can give a certain indirect measure of the force exerted by the individual muscle and of the time of its activation

The first electromyographic studies of the muscles of mastication concerned their action during the basic mandibular movements (*Moyers* 1950 *Carlsoo* 1952 1956 a and b *Gopfert* and *Gopfert* 1955 *Zenker* and *Zenker* 1955 *Hickey* et al 1957 *Surila* 1958 *Eschler* 1958 *Ekholm* and *Surila* 1960 *Woelfel* et al 1960 *Surila* et al 1960) These studies confirmed the generally accepted concept of the action of the muscles based on their anatomy The literature is reviewed below as it concerns bite mastication swallowing postural activity and the relation between the activity in the muscles of mastication and the morphology of the chewing apparatus

Bite Recordings obtained during maximal bite (*MacDougall* and *Andrew* 1953 *Greenfield* and *Wyke* 1956 *Latif* 1957 *Woelfel* et al 1960 *Okun* 1960) showed that the elevator muscles were activated differently according to where the mandible was placed in the sagittal plane as compared with bite in the intercuspal position *) biting on the incisors involved activation mainly of the masseter muscles whereas biting in a retruded position involved substantial activity in the posterior temporal muscles According to *Garrett* et al (1964) biting with the jaws separated by 7 to 27 mm caused activity in the masseter muscles to diminish about 40 per cent though the biting pressure was unchanged (1–7 kilograms)

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*) Intercuspal position the intermaxillary relationship characterized by the interdigitation of the cusps and occluding sulci of the occlusal reliefs (*Posselt* 1952)

Mastication During unilateral chewing the ipsilateral masseter and temporal muscles were activated more than the contralateral (*Perry* and *Harris* 1954 *Perry* 1955 1961 *Pruzansky* et al 1958 *Ahlgren* 1966) The ipsilateral temporal muscle was activated sooner than the masseter whereas the masseter muscle was the first to be activated on the contralateral side (*Perry* and *Harris* 1954 *Perry* 1955 1961) As to the other muscles of mastication the external pterygoid muscles showed most activity on the balancing side (*Hickey* et al 1963) The time relationship between muscle activity and tooth contact during natural chewing is reported divergently *Møller* (1960) and *Graf* and *Zander* (1964) found that the duration of contact outlasted activity in the elevator muscles whereas the figure of *Scharer* and *Stallard* (1965 Fig 3) indicates that muscle activity and tooth contact coincided Electromyographic recordings from the temporal masseter (*Neuman* 1950 *Eschler* 1955 *Ahlgren* 1966) and suprahyoid muscles (*Kawamura* 1957 a *Eschler* 1958 *Kraner* 1960) during natural chewing of various foods have been evaluated quantitatively However only *Ahlgren* (1966) considered the variation about the mean of the values during natural chewing of peanuts he found the same degree of activity in the right and left temporal and masseter muscles

Swallowing *Tulley* (1953) and *Baril* and *Moyers* (1960) studied the muscles of the lips and the elevator muscles of the jaw during swallowing In a number of their subjects the pattern of activity conformed neither to the normal nor to the abnormal patterns observed clinically by *Rix* (1946) and *Gwynne Evans* (1954) thus these patterns cannot serve as basis to classify the muscle activity during swallowing *Findlay* and *Kilpatrick* (1960) showed that activity during swallowing in the temporal and masseter muscles differed significantly in amount and duration from subject to subject whereas the differences between the right and left muscles of a pair were not significant *Graf* and *Zander* (1964) found that tooth contact in the intercuspal position was maintained for a considerable time during swallowing whereas *Scharer* and *Stallard* (1965 Fig 5) found that tooth contact involved a sliding movement and that the intercuspal position was only attained for a short interval

Postural activity Study of postural activity in the muscles of mastication has aimed to answer two main questions

1 Is there activity in the muscles of mastication when the mandible is at rest? *Shpuntoff* and *Shpuntoff* (1956) and *Jarabak* (1957) found no activity while *Moyers* (1949) *Carlsoo* (1952) *Gopfert* and *Gopfert* (1954) *Latif* (1957) *Kawamura* (1957 b) and *Sirila* (1958) found activity in the temporal muscles

2 Is it possible to determine the rest position of the mandible by the electromyogram? *Mullen* (1956) *Roberts* (1960) *Hickey* et al (1961), and *Krajicek* et al (1961) found least muscle activity when the jaw was in its resting position *Gopfert* and *Gopfert* (1954) and *Garnick* and *Ramfjord* (1962) reported that activity in the temporal and masseter muscles decreased when the mandible was lowered beyond the clinical rest position

Relation of muscle activity to the morphology of the chewing apparatus In subjects with malocclusion of the type Angle Class II, Division 1 *Moyers* (1949) found predominance of the posterior part of the temporal muscle when the mandible was at rest (subjects with mandibular retrognathism) and during mandibular movements (subjects with mandibular retrognathism and total or alveolar maxillary prognathism) The coordination of the masseter and temporal muscles during maximal bite depends on where the mandible is placed in the sagittal plane (cf p 14) and electromyograms during maximal bite have been used to indicate the sagittal relation of the mandible to the maxilla in subjects with malocclusion (*Grossmann* and *Greenfield* 1956 *Timms* 1960 *Grossmann* et al 1961, *Timms and Greenfield* 1961) *Perry and Harris* (1954) and *Perry* (1955 1961) found the masseter muscles in unilateral chewing to be activated earlier in individuals whose malocclusion was of the type Class II Div 1 than in subjects with normal occlusion With 7 mm bite opening and biting pressures of 1 2 2 2 and 3 4 kg and with 23 mm opening at pressures of 1 2 and 2 2 kg *Garrett* et al (1964) observed more activity in the masseter muscle in a group of Class II subjects than in subjects with normal occlusion. In other electromyographic studies (*Findlay* and *Kilpatrick* 1960 *Krazer* 1960 *Baril* and *Moyers* 1960 *Watt* 1964 *Ahlgren* 1966) as well as in studies of the force exerted by the elevator and lip muscles (*Friel* 1924 and 1926 *Lancet* 1927 *Winders* 1958 and 1962 *Kydd* et al 1963 *Kydd* and *Neff* 1964 *Werner* 1964) there was no evidence of a difference in muscle activity between subjects with normal and malocclusion.

As to the shape of the mandible *Watt* (1961 1963) reported that a small gonial angle was associated with strong masticatory activity in the masseter muscle especially in its anterior part *Ahlgren* (1966) found a tendency of a similar association (a weak negative correlation) between the activity both in the temporal and masseter muscles during chewing and the gonial angle

ELECTROMYOGRAPHIC METHODS

The electrical activity of muscles can be recorded with surface or needle electrodes Two types of *surface recording* have been used with bipolar recording two identical electrodes are placed over the muscle With unipolar recording one electrode is placed over the muscle and the other indifferent" electrode at some distance where electrical activity is slight or absent. Activity below the "indifferent" electrode is a source of error in the interpretation of the activity attributed to a given muscle *Hickey* et al (1958) reduced this interference by using a five point reference electrode similar to the common : reference electrode used in electroencephalography (*Offner* 1950 *Goldman* 1950)

Intramuscular recordings can be obtained with different types of electrodes

1 Unipolar needle or wire electrodes with a remote indifferent electrode in the muscle or on the surface
2 Concentric needle electrodes leading off between the core and the cannula (*Adrian* and *Bronk* 1929)
3 Bipolar needle or wire electrodes leading off between two separately inserted needles or wires
4 Bipolar needle electrodes leading off between two cores in a cannula less than 0 5 mm apart.
5 Multi lead needle electrodes using one of the leads or the cannula as indifferent" electrode (*Buchthal* et al 1957 a)

The unipolar technique with a remote "indifferent" electrode presents the same source of error in intramuscular as in surface recording The bipolar electrode (4) causes a "differentiation" of the single action potential (*Buchthal* et al 1954) and results in a pick up too localized for the study of the action potential pattern of a whole muscle The pick up of the concentric needle electrode (2) is less localized and this electrode is the most suitable of the standard needle electrodes (2 4 and 5) for the study of muscle coordination by intramuscular recording. Recording with a concentric needle electrode corresponds closely to unipolar recording with the cannula as indifferent" lead (*Buchthal* et al 1954)

The action potentials picked up by the electrodes are usually amplified by difference amplifiers which transmit the potential difference between two leads and reject a common voltage to earth. The accuracy with which the signal is reproduced and the effectiveness of rejection are influenced by the impedance of the electrodes the input impedance of the amplifier and the frequency response of the recording system with electrodes (*Buch*

thal et al 1954) These physical variables have received little attention in previous studies of the coordination of the muscles of mastication *Greenfield* and *Wyke* (1956), *Kawamura* (1957 b) *Latif* (1957), *Kydd* (1959) *Liebman* and *Cosenza* (1960), *Findlay* and *Kilpatrick* (1960) and *Baril* and *Moyers* (1960) measured the resistance of surface electrodes by means of a dc-circuit but did not take into account the capacitive component of the impedance of the electrodes *Stacy* et al (1958) analysed the frequency components of surface electromyograms and found that their distribution had its maximum between 100 and 200 cycle/sec

STATEMENT OF THE PROBLEM

The present study deals with the electrical activity in the muscles of mastication in young adult males during chewing and swallowing during full effort and at rest (postural activity) The muscles investigated were the temporal masseter internal and external pterygoid, digastric mylohyoid and the superior and inferior orbicularis oris An attempt was made

1 To evaluate the effect of the impedance of the electrodes of the input impedance of the amplifier and of the frequency response of the recording system on electromyographic recordings from the muscles of mastication
2 To describe quantitatively the coordination of the muscles and the degree and the duration of the activity in each muscle
3 To describe the relation in time between muscle activity and tooth contact during chewing and swallowing
4 To analyse the correlation between the electrical activity in the muscles and the morphology of the cranial base the jaws and the dental and alveolar arches

All data were analysed statistically with respect to variation from subject to subject and with respect to reproducibility in the same subject

MUSCLES OF MASTICATION
GROSS ANATOMY AND PLACEMENT OF ELECTRODES*)

Elevator muscles

The temporal muscle (M temporalis Fig 1)

The temporal muscle arises from the temporal fossa and the deep surface of the tem

*) The anatomical description is based on *Schumacher* (1961) *Davies* and *Davies* (1962) and *Sicher* (1965) The designation in parenthesis refers to *Nomina Anatomica* (1961)

poral fascia The muscle is bipennate its fibres converging in the sagittal and the frontal planes and ending in a tendon which passes medially to the zygomatic arch (Fig 2) The tendon is inserted into the coronoid process of the mandible on the medial side of which it divides one tendon following the anterior border of the ramus the other the temporal crest

The temporal muscle acts mainly as an elevator of the mandible but is also a retractor and may then possibly act in dorsal translation and during transverse rotation toward the ipsilateral side In addition *Schumacher* (1961) assumes that the fibres originating from the retro-orbital area act to protrude the mandible

The two parts of the temporal muscle were studied separately the fibres of the anterior part lying vertically and those of the posterior part horizontally The electrical activity was recorded bilaterally and picked up by means of bipolar surface electrodes (see p 15)

The masseter muscle (M masseter Fig 1)

The masseter muscle is divided in two portions (pars superficialis pars profunda) The larger and superficial portion arises from the anterior two thirds of the lower border of the zygomatic arch the fibres being directed postero inferiorly to insert with the deep portion The deep portion (divided in two layers by *Davies* and *Davies* 1962) arises from the posterior third of the lower border and the entire deep surface of the zygomatic arch The posterior fibres lie vertically the anterior fibres fuse with those of the superficial portion and are directed postero inferiorly The fan shaped deep portion of the masseter muscle is covered by the superficial portion except for the most posterior part in front of the joint capsule

The masseter (Fig 2) is multipennate with five intramuscular tendons three attached to the zygomatic arch two to the ramus of the mandible The superficial and deep portions of the muscle intermix and are inserted together into the ramus of the mandible as far anteriorly as the level of the distal surface of the second molar posteriorly they cover the mandibular angle Caudo-cranially the insertion extends from the

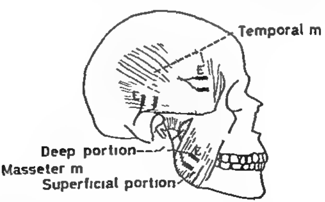

Fig 1

Position of surface electrodes (E) *above the anterior and posterior part of the temporal and the masseter muscles* (redrawn from *Sicher* 1965)

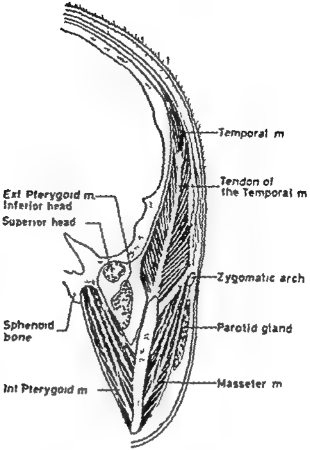

Fig 2

Frontal section through the right half of the skull the muscles of mastication and the tendons (from *Schumacher* 1961)

lower border of the mandible to the base of the coronoid process leaving only th condylar process uncovered The most medial fibres of the deep portion of the massete muscle fuse with the superficial fibres of the temporal muscle According to *Carlso* (1952) and *Schumacher* (1961) 25–30 per cent of the force exerted by the massete to close the jaw may be exerted to protrude it *Carlsoo* (1952) and *Sicher* (196 assumed the vertical fibres of the deep portion to act as retractors

The electrical activity from the masseter muscle was recorded bilaterall by means of bipolar surface electrodes placed over the fleshy body of th superficial portion No attempt was made to differentiate between the func tion of the superficial and of the deep portions (Fig 1)

The internal pterygoid muscle (M pterygoideus medialis Fig 3 A)

The internal pterygoid muscle arises mainly from the medial surface of the latera pterygoid plate and from the grooved surface of the pyramidal process of the palatin

bone In addition it arises superficially from the lateral surface of the pyramidal process of the palatine bone and from the tuberosity of the maxilla (Fig 3 B) The superficial origin covers the lower head of the external pterygoid muscle inferiorly The muscle is multipennate with 6–8 intramuscular tendons (Fig 2) The fibres run downwards and backwards and are inserted into the medial surface of the ramus and the angulus of the mandible The area of insertion is delineated by the lower half of the posterior border of the ramus the mandibular foramen and the mylohyoid groove *Sicher* (1965) believes that the internal pterygoid muscle is active only to close the jaw whereas *Carlsoo* (1952) and *Schumacher* (1961) assume it to be a protractor as well

The electrical activity was recorded bilaterally by means of concentric needle electrodes (see p 15 no 2) with a length of 30 or 42 mm inserted from below and just in front of the angulus of the mandible (Fig 3 A) The position was changed until maximal response was obtained during clench

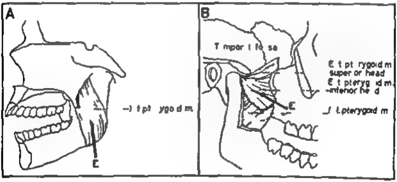

Fig 3

Position of concentric needle electrodes (E) *in the internal* (A) *and the external* (B) *pterygoid muscles* (redrawn from *Sicher* 1965 (A) and *Schumacher* 1961 (B))

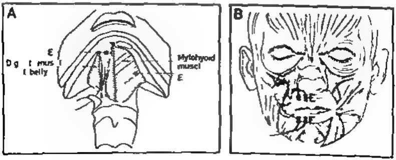

Fig 4

A Position of concentric needle electrodes (E) *in the digastric and mylohyoid muscles*
B Position of surface electrodes (E) *above the muscles of the upper and lower lips* (redrawn from *Sicher* 1965)

ing of the teeth Usually several insertions were required until the needle recorded a maximal response without causing discomfort The cable of the electrode was fixed to the skin by adhesive tape Chewing caused artefacts from movement of the electrode when it had been placed by the intraoral approach

Depressor muscles

The external pterygoid muscle (M pterygoideus lateralis Fig 3, B)

The external pterygoid muscle arises by two heads an upper from the infratemporal surface and the infratemporal crest of the greater wing of the sphenoid bone a lower from the lateral surface of the lateral pterygoid plate The fibres converge postero laterally toward the condyle The upper head is covered by the deep fibres of the temporal muscle and the anterior part of the lower head is covered by fibres from the internal pterygoid muscle The fibres from the two heads fuse close to the point of insertion but the lower head is mainly inserted into the pterygoid fovea the upper head is inserted into the medial surface of the articular capsule and thus indirectly in the articular disc The structure (Fig 2) is less complicated than in the elevator muscles

The action of the external pterygoid muscle is forward translation and contralateral transverse rotation Since the pull of the muscle bisects the intercondylar axis (*Carlsoo* 1956 b) it does not contribute to the hinge movement It is active when the condyles slide forward during opening of the jaw *Davies* and *Davies* (1962) in contrast to *Sicher* (1965) assume the action of the external and internal pterygoid muscles to be synergistic during protrusion

The electrical activity was recorded bilaterally with concentric needle electrodes 30 mm long inserted intraorally just lateral to the maxillary tuberosity in a postero-superior direction. Thereby the muscle was entered halfway between its origin and insertion and the internal pterygoid (below) and the temporal muscles (above) were avoided No attempt was made to differentiate the activity of the two heads but recordings were probably obtained from the lower While the mandible was protruded the position of the electrode was adjusted until the response obtained was maximal Then the electrode cable was fixed with a ligature around the neck of the second premolar taken out through the corner of the mouth and fastened with adhesive tape to the cheek (*Moyers* 1949 *Carlsoo* 1956 b) An extraoral approach through the semilunar notch of the mandible (*Ekholm* and *Siirila* 1960) interfered with chewing because the needle penetrated the tendons of the masseter muscle

The digastric muscle (M digastricus venter anterior venter posterior Fig 4 A)

The digastric muscle consists of two bellies connected by a strong tendon The posterior belly is attached to the mastoid notch medial to the mastoid process the anterior belly

is attached to the digastric fovea of the mandible close to the midline and the base of the mandible The intermediate tendon is fixed to the body of the hyoid bone by a fibrous loop

Connected to two movable bones the mandible and the hyoid the action of the digastric muscle depends on the fixation of either or both of these When the hyoid bone is fixed in its lowest position the digastric muscle depresses the mandible When the mandible is fixed the digastric muscle elevates the hyoid bone activation of one of the two bellies causes the hyoid bone to move forward (ant belly) or backward (post. belly)

The electrical activity was recorded bilaterally from the anterior belly of the digastric muscle and was led off with concentric needle electrodes 20 mm long The mandible was lowered against resistance the muscle palpated and the electrodes placed 2 cm behind the chin at equal distances from the mid line When the response obtained was optimal the electrode cable was fastened to the lower border of the mandible

The mylohyoid muscle (M mylohyoideus Fig 4 A)

The mylohyoid muscle arises from the mylohyoid line of the medial surface of the body of the mandible and extends from the last molar almost to the midline The fibres ar inserted into the anterior surface of the hyoid bone and into the mylohyoid raphe The direction of the fibres is medial and downward the posterior fibres lying almost vertically

According to *Sicher* (1965) the anterior fibres have little influence on the position of the mandible and are mainly concerned with the vertical position of the tongue The posterior fibres run vertically and pull the mandible downwards when the hyoid bone is fixed The mylohyoid muscle (as well as the digastric muscle) is assumed to take part in elevation of the tongue during swallowing

The electrical activity was recorded bilaterally by means of concentric needle electrodes 30 mm long The electrodes were inserted just medially to the lower border of the mandible about 4 cm behind the chin The mu cle was approached laterally to avoid contact with the digastric muscle and distally where the muscle increases in thickness Search for maximal response was made while the subject pressed his tongue against his palate then resistance to the penetrating needle indicated the position of the muscle Although the muscle was easy to locate it was difficult to keep the electrodes in place since the muscle is thin

The muscles of the lips

The orbicularis oris muscle (M orbicularis oris pars marginalis pars labialis Fig 4 B)

The orbicularis oris muscle is the oral sphincter and occupies the entire width of the upper and lower lip the fibres being divisible into a labial and marginal portion It consists in

part of fibres from other facial muscles The fibres of the orbicularis oris proper are oblique passing from the deep surface of the skin to the mucous membrane The buccinator muscle contributes to the deeper part of the orbicularis oris the middle fibres decussate at the corner of the mouth the peripheral fibres passing uncrossed into the lips The superficial fibres from the levator and depressor anguli oris cross each other at the angle of the mouth and insert into the skin of the lower and upper lips respectively Fibres are also derived from the levator labii superioris the depressor labii inferioris the zygomaticus major the mentalis and the incisivus labii superioris and inferioris

Activity in the orbicularis oris muscle was recorded from the upper and lower lips The surface electrodes were placed on the right side close to the midline (Fig 4 B) The electrodes on the upper lip were placed on the area delineated by the midline the naso-lacrimal groove and the prolabium the electrodes on the lower lip were situated between the labio-mental groove and the prolabium

Innervation

The muscles studied are supplied by the third division of the trigeminal nerve except for the orbicularis oris which is innervated by the facial nerve

ELECTROMYOGRAPHY

In man the electrical activity of muscle during voluntary contraction was first demonstrated by *Du Bois Reymond* (1849) one finger on each hand of the subject was connected to the leads of a needle galvanometer As each arm was flexed there was a deflection of the needle The degree of deflection increased with the strength of contraction was larger when the right arm was flexed in right handed subjects increased when the epidermis was removed and decreased at the onset of fatigue *Piper* (1907) using a string galvanometer and *Buchanan* (1908) with a capillary electrometer led off action potentials in the muscles of the forearm and in the masseter by surface electrodes and recorded them on photographic paper They found that contraction was associated with potential changes which increased in amplitude with the strength of contraction

The motor unit

The unit of voluntary and reflex activity is the group of fibres innervated by the same motor neurone (*Lidell* and *Sherrington* 1925 *Porter* 1930) A motor neurone its axon

and the muscle fibres which it innervates are termed a *motor unit* (*Lidell* and *Sherrington* 1925)

According to histological and electrophysiological evidence (*Wohlfart* 1949 *Buchthal* et al 1957 a) the fibres of a motor unit are divided in groups—subunits—of up to 30 fibres The electrophysiological evidence of subunits—the volume conduction of the spike potential as determined by multilead electrodes (*Buchthal* et al 1957 a)—has been questioned by *Ekstedt* (1964) He claims (1) that the spike potential originates from a single fibre and (2) that *Buchthal* et al (1957 a) calculated the spread of the spike potential and therefore the diameter of its source too large due to capacitive coupling between the leads of their electrode *Buchthal* and *Rosenfalck* (1966) have refuted this interpretation and explain the steeper decrease of the voltage of the spikes observed by *Ekstedt* (1964) as being due to recording from peripheral subunits According to *Buchthal* et al (1957 b and 1959) the subunits of each motor unit are scattered over an area with a diameter of 4–7 mm in the muscles of the upper extremities and of 7–10 mm in the lower extremities

The number of muscle fibres per motor unit differs in various muscles 5 in the rectus oculi ext muscle (*Torre* 1953 no correction for small nerve fibres and sensitive fibres) 100–300 in the intrinsic laryngeal muscles (*Faaborg Andersen* 1957) 600–900 in the masseter and temporal muscles (*Carlsoo* 1958) In the limb muscles the motor units may have 1900 fibres (gastrocnemius muscle *Feinstein* et al 1954)

Carlsoo (1958) found a mean fibre diameter in the temporalis muscle of 20 μ and a cross sectional area of the muscle fibres of one motor unit of 0 29 mm The corresponding figures for the masseter muscle were 21 μ and 0 22 mm With a maximal tetanic force of the muscle fibres of 4 kg cm (*Ramsey* and *Street* 1940 frog muscle 3 5 kg/cm *Haxton* 1944 ankle flexors in man 3 9 kg/cm) a single motor unit can produce a maximal isometric force of 12 grams in the temporal muscle and 9 grams in the masseter muscle In the gastrocnemius muscle of the cat the individual motor units produce maximally 47 grams (average) the tension developed from a single twitch being about 6 per cent of the tetanic tension (*Wuerker* et al 1965)

Electrical activity in relaxed muscle

It is generally accepted that action potentials are absent in relaxed muscle (*Piper* 1907 *Adrian* and *Bronk* 1929 *Smith* 1934 *Clemmesen* 1951) Only *Gopfert* (1952) recorded action potentials of 2–3 μV from relaxed muscle He considered these potentials a sign of the motor unit activity responsible for muscle tone *Joseph* et al (1955) demonstrated that the small potentials persisted when the electrodes were placed on bone As even the slightest contraction resulted in large spike potentials they concluded that the small potential changes were not caused by motor unit activity As possible origins of these potentials *Joseph* et al (1955) suggested irregular flow of ions in the blood vessels thermal noise or endplate activity Non propagated endplate potentials (miniature potentials" *Fatt* and *Katz* 1950 1952) have been recorded in relaxed human muscle in situ (*Rosenfalck* and *Buchthal* 1962)

Gradation of muscle activity

In the early studies of muscle action potentials the electrodes were placed on the skin or pairs of needles were inserted into the muscle To obtain localized recording *Adrian* and *Bronk* (1929) introduced the concentric needle electrode consisting of an insulated

wire within a cannula. The potentials are led off between the cannula and the bared tip of the inner core. With this electrode they recorded action potentials from muscle fibres in the immediate vicinity of the tip which discharged with a frequency of 5 to 25 per sec during weak activity. Since single nerve fibres in cats discharged at similar frequencies *Adrian* and *Bronk* (1929) concluded that each action potential in muscle reflected the firing of a single motor unit. During stronger contraction the frequency of discharge of the individual motor unit increased and new units were recruited, their action potentials distinguishable from the first by differences in frequency and amplitude. During moderate and strong contraction the electromyogram appeared as an interference pattern in which the individual potentials could no longer be discriminated.

When leading off between two wires in a cannula (bipolar electrode) the pick up was still more localized and the firing of a single motor unit could be followed to rates of up to 50 per sec during strong contraction; the frequency range was usually between 10 and 25 per sec (*Adrian* and *Bronk* 1929). As the frequencies were seldom so high as to produce complete fusion of the contractions, smooth contraction was considered to be due to the asynchronous action of different motor units.

The frequency range of the individual motor unit demonstrated by *Adrian* and *Bronk* (1929) was confirmed by *Bigland* and *Lippold* (1954 b) in experiments with electrical stimulation and voluntary contraction. They never found frequencies exceeding 50 per sec and this upper limit was reached only when the contraction was more than 75 per cent of maximum. At tensions between 5 and 75 per cent of maximum, the frequencies ranged between 25 and 35 per sec, and were not identical in different motor units. Some units would commence at a certain level of contraction and fall out again at a higher level. *Dasgupta* and *Simpson* (1962) observed that the frequency increased with tension to an upper limit of about 20 per sec (first dorsal interosseous muscle). The firing rate of the motor units in the muscles of mastication has not been studied.

The motor unit potential

The basic element of the motor unit potential is the potential of the single muscle fibre. The action potential of the isolated single fibre recorded extracellularly is diphasic and has a duration corresponding to the spike of the intracellular action potential (*Håkansson* 1957). Recorded in situ the extracellular potential of a single fibre is often triphasic, the third positive phase reflecting the first phase of repolarization of the membrane (*Rosenfalck* 1960).

The extracellular potential of a single fibre is usually considered to be represented by the fibrillation potentials of denervated muscles (*Denny Brown* and *Pennybacker* 1938). Although later investigations have shown that some fibrillation potentials originate from the fully synchronized firing of a small group of muscle fibres (*Rosenfalck* and *Buchthal* 1960), the duration of fibrillation potentials indicates a 1–5 msec duration of the action potential of single fibres.

Within each subunit (see p. 23) of the motor unit the action potentials of single fibres summate and give rise to a subunit potential. The amplitude of this potential depends largely on the distance between the recording electrode and the subunits closest to the electrode, the amplitude being reduced by 90 per cent at a distance of 0.5 mm from the source (*Buchthal* et al. 1957 a). The longer duration of motor unit as compared with fibrillation potentials is due to the spatial distribution of the endplates which causes temporal dispersion of the potentials of the fibres of the motor unit (*Buchthal*

et al 1957 b) Due to the steep decrease in spike amplitude with increasing distance to the electrode only a fraction of the subunits belonging to a motor unit contributes to the motor unit potential recorded at a given point Hence each motor unit has many motor unit potentials (*Buchthal* 1957 a) In the muscles of mastication (mas eter and temporal muscles) the motor unit potentials have a duration of 4–15 msec and most are di and triphasic (*Carlsoo* 1958) I found that the peak voltage of 330 motor unit potentials from the temporal muscles of III subjects (males 23–26 years old) varied between 50 and 8000 μV (average 273±15 μV standard deviation 270 μV) in the masseter muscles of the same subjects the peak voltage varied between 40 and 3000 μV (average 271±12 μV standard deviation 224 μV 332 potentials) Such a wide variation has been found in other muscles (*Buchthal* 1957 b)

Electromyogram and mechanical response

When one wishes to relate the electrical and the mechanical response of a muscle one must take into consideration that the mechanical response occurs later than and persists after the corresponding electrical discharge (human muscle *Inman* et al 1952 *Desmedt* 1958) Furthermore the mechanical response depends on the stretch and load of the muscle 1) The force which the muscle can develop during contraction depends on the length of the muscle Maximal force is developed when the muscle is near its resting length (frog muscles *Blix* 1895 human muscles *Ralston* et al 1947) in the case of the muscles of mastication probably the length with the mandible at rest position The decrease in tension at lengths above resting length is to a large extent compensated for by the passive elastic force of the muscle 2) The degree and velocity of shortening depend on the initial length of the muscle and on the load (frog muscle *Hill* 1939 human muscle *Hill* 1940 *Ralston* et al 1949) 3) The maximal force a muscle can exert is a function of the total number of fibres (the physiological cross section) whereas maximal shortening depends upon the length of the fibres

The electromyogram and tension The strength of voluntary contraction is increased both by an increase in the rate of discharge of the individual motor unit and mainly by recruitment of more motor units Since each action potential represents activation of a motor unit a correlation can be expected between the force of the muscle and the pattern of electrical activity

The electromyogram and shortening When shortening takes place the relation between the electrical and mechanical response of muscle becomes more complicated The velocity of shortening is the less the smaller the number of activated motor units As shortening proceeds the active motor units take over the load initially carried by the inactive units causing the velocity of shortening to decrease with the degree of shortening

Evaluation of the electromyogram

A concentric needle electrode may pick up simultaneously the spike potentials of 40 subunits belonging to 10–30 intermingled motor units (*Buchthal* and *Rosenfalck* 1963). When contraction is weak and the active motor units are few and scattered, their spike potentials can be counted over a certain time to evaluate the degree of electrical activity. With increasing strength of contraction more motor units are activated; since they discharge asynchronously (25–35 times per sec, *Bigland* and *Lippold* 1954 b) the subunit potentials in the immediate vicinity of the tip of the electrode summate to produce an interference pattern whose amplitude increases with the strength of contraction. During maximal effort the subunit potentials occur at average intervals corresponding to the duration of their peaks and summation takes place continuously (*Buchthal* and *Rosenfalck* 1963). Since surface electrodes record the activity from a larger area than do needle electrodes, they pick up more potentials during weak effort. Due to the amplitude–distance relationship (*Buchthal* et al. 1957 a, see p. 24) the amplitude of these potentials varies less than in recordings with needle electrodes. Hence surface electromyograms during weak effort consist of numerous small potentials and usually the action potentials of different motor units cannot be distinguished.

In natural function the electromyogram presents an interference pattern. The peak-to-peak amplitude, or the area of this pattern, provides a measure of the force developed by the muscle or of the velocity of shortening. Similarly a count of the number of potentials provides a measure of the number of active motor units.

The amplitude of the interference pattern has been estimated by

1) summation of the action potentials by means of a ballistic galvanometer (*Stetson* and *Bouman* 1933)
2) summation of the action potentials through a thermo-cross (*Wegener* 1941)
3) determining the average peak-to-peak voltage over a certain time interval (*Inman* et al. 1944)
4) readings from a strongly damped voltmeter (*Bayer and Flechtenmacher* 1950)
5) graphic integration (*Lippold* 1952)
6) electronic integration (*Bates* and *Cooper* 1954)
7) continuous mean voltage recording through an RC filter (*Inman* et al. 1952) or an RLC filter (*Sten Knudsen* 1957)

Procedure 1 gives a measure of the total amount of electrical activity but not of its time course Procedures 2–4 are not suitable unless the activity remains constant for some time The total amount of electrical activity displayed during a certain interval of time can be measured by integration (5 and 6) In addition the slope of the trace reflects the charging of integrators (6) and may provide information as to the instantaneous intensity and variation of the electrical activity However when the changes in potential are rapid and large as in chewing the frequent discharges of the integrator make an evaluation of the time course of the activity time-consuming and difficult. Therefore I considered the continuous recording of the numerical mean voltage through an RC or RLC filter to be a more suitable method for my purposes. The numerical mean voltage determined through the RLC filter was a more correct measure of the true numerical mean voltage than that obtained through the RC filter (*A Rosenfalck* 1959)

Mean voltage and mechanical activity of muscle The mean voltage varies linearly as a function of isometric tension (*Buchthal* 1942 *Snyder* et al 1949 *Bayer* and *Flechtenmacher* 1950 *Lippold* 1952 *Inman* et al 1952 *Knowlton* 1956 *Sten Knudsen* 1957 *Scherrer* et al 1957 *Lenman* 1959 *Ahlgren* 1966) The more than linear increase in mean voltage with load observed by some authors (*Wegener* 1941 *Inman* et al 1944 *Geltzer* 1953 *Zwemer* 1955 *Angelone* et al 1960 and *Garrett* et al 1964) may be explained by the effects of fatigue During constant load the mean voltage increased with fatigue but at each level of fatigue the relation between the electrical activity and tension was linear (*Edwards* and *Lippold* 1956) The slope of the relation between mean voltage and load was significantly steeper when exhausted muscles were recorded from indicating that more motor units were required to carry a given load

During free rapid movements of the fingers the ratio of velocity to summated action potentials from the muscles of the forearm (ballistic galvanometer) was constant and the summated action potentials from flexor and extensor muscles were proportional to the length of the excursion (*Stetson* and *Bouman* 1933) During changes in length at the same velocity the electrical activity was directly proportional to the load the slope of the relation being the steeper the faster the shortening during shortening at constant load the electrical activity increased linearly with the velocity while it remained constant during lengthening (*Bigland* and *Lippold* 1954 a)

Opinions differ with regard to the applicability of *frequency counts* to estimate the degree of mechanical muscle activity In the muscles of the forearm the spike frequency of the interference pattern remained unchanged

in spite of variations in tension (*Wachholder* 1923 *Richter* 1927) Similarly the spike frequency of the interference pattern in the temporal and masseter muscles remained constant in spite of increasing force of biting (*Geltzer* 1953) and in spite of increasing loads on the mandible (*Zwemer* 1955) On the other hand *Bergstrom* (1959) and *Close* et al (1960) found a linear relation between the number of spikes and the mechanical activity of the muscle

Finally in the brachial triceps muscle *Willison* (1963 1964) found that *the frequency as well as the amplitude* of the potential changes increased with the load (0 5–2 0 kilograms)

Influence of electrode and amplifier on the accuracy of electromyographic recordings

The accuracy with which action potentials are recorded is influenced by the impedance of the electrodes the input impedance and frequency response of the amplifier and the effectiveness of rejection of common voltages (*Buchthal* et al 1954) The amplitude of the action potentials may be reduced due to *voltage division* between electrode and amplifier (Fig 5) The significance of voltage division may be seen from the effect of different values of the ratio impedance of the electrode to input impedance of the amplifier if for instance this ratio is 1 0 25 or 0 01 the reduction of the amplitude amounts to 50 20 or 1 per cent *Interference from common voltages* could be avoided if voltage division was equal on both inputs of the difference amplifier (Fig 5 Za = Zb) Since the impedances of electrodes are unstable and vary widely the interference from common voltages depends on the voltage division at the electrode of highest impedance (Fig 5 Za > Zb *Guld* 1961) Hence the rejection of common voltages demands a high input impedance of the amplifier as compared to the impedance of the electrode

The influence of the impedance of the electrodes the input impedance and frequency response of the amplifiers and the rejection of common voltages on the mean voltage depends on the frequency components of the electromyogram The upper and lower limits of these components therefore represent the range within which the effect of the physical variables must be considered With regard to the amplitude of single action potentials an upper frequency limit of the recording circuit below 10^4 cycle/sec reduces the amplitude of the spike (*Buchthal* et al 1954) Surface electromyograms from the muscles of mastication contained frequencies of at most 600 cycle/sec these were few and the frequency distribution had its peak at 100–200 cycle/sec (*Stacy* et al 1958) *Kaiser* and *Petersen* (1963) studied the fre

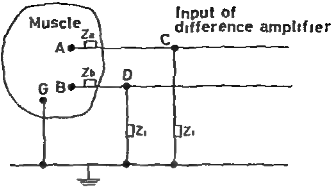

Fig 5

To illustrate the effect of impedance of the electrodes and input impedance of the amplifier on voltage division and interference from common voltages A and B are *single leads of a recording electrode connected to the input of a difference amplifier* at C and D Za and Zb are impedances of the electrodes (including tissue impedance) Z_i = the input impedance of the difference amplifier (assuming symmetrical inputs) and *G the earth electrode*

Voltage division If A has a potential to earth V the voltage drop across the impedance Za results in a potential V_{a_i} at the input (C) of the amplifier thus

$$V_{a_i} = V_a \left(1 - \frac{Za}{Zi + Za}\right) \qquad \text{(I)}$$

the voltage reduction being proportional to $\dfrac{Za}{Zi + Za}$

Similarly a potential V_b at B at the input (D) is reduced to

$$V_{b_i} = V_b \left(1 - \frac{Zb}{Zi + Zb}\right) \qquad \text{(II)}$$

Therefore the potential difference between A and B is recorded as

$$V_{a_i} - V_{b_i} = V_a \left(1 - \frac{Za}{Zi + Za}\right) - V_b \left(1 - \frac{Zb}{Zi + Zb}\right) \qquad \text{(III)}$$

Interference from common voltages The interference V_g from a common voltage V_g can be derived from (III) ($V_a = V_b = V_g$)

$$V_{g_i} = - V_g \left(\frac{Za}{Zi + Za} - \frac{Zb}{Zi + Zb}\right) \qquad \text{(IV)}$$

With identical impedances of the electrodes a common voltage is rejected completely (Za = Zb) *if A represents the core of a concentric needle electrode with Za >> Zb* the interference from common voltage is about

$$V_{g_i} \approx - V_g \frac{Za}{Zi + Za} \qquad \text{(V)}$$

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Chapter II

METHODOLOGICAL INVESTIGATIONS

The numerical mean voltage of the electromyogram was used as a measure of the number of active motor units and of their frequency of firing i e as a measure of the motor innervation of the muscle To record the action potentials without distortion and without interference from potentials of neighbouring muscles requires the following conditions of recording

1) The upper limiting frequency of the amplifiers and of the recording system must be high enough to allow amplification without distortion of the spike components of the electromyogram

2) The input impedance of the amplifier must be high as compared to the impedance of the electrodes Thereby a reduction of the amplitude of the action potentials is avoided which otherwise would be caused by the voltage division between electrode and amplifier

3) The rejection of common voltage must be large enough to prevent extraneous activity from affecting the recordings

Whenever possible surface recording was applied since it interferes least with natural function Surface leads required investigations of the extent to which potentials from neighbouring muscles interfered with the electromyogram of the muscle under study In muscles not accessible to surface recording the electrical activity was recorded by concentric needle electrodes Finally to decide to what extent the recording from one site reflects the pattern of activity of the whole muscle information was required as to the distribution of activity within the muscle

In summary the following methodological data were collected

A The influence of the impedance of the electrodes the input impedance and frequency response of the amplifier and the rejection of common voltage on the mean voltage

B Comparison of bipolar and unipolar surface recording and recording with concentric needle electrodes

quency distribution of electromyograms (intramuscular recording) during strong voluntary contractions in the orbicularis oris and the brachial biceps muscles Between 25 and 10 000 cycle/sec the distribution rose from 25 to 100 cycle/sec had its peak between 100 and 400 cycle/sec and decreased rapidly above 800 cycle/sec Only in one experiment did they find substantial activity above 6000 cycle/sec Hence the high frequency components of the single action potential (*Buchthal* et al 1954) play a minor role in the frequency distribution of the interference pattern and cannot be expected to have significant influence on the mean voltage Also frequencies of less than 50 cycle/sec contributed only little to the interference pattern and it seemed sufficient to study the frequency range from 20 to 10 000 cycle/sec

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B Comparison of bipolar and unipolar surface recording and recording with concentric needle electrodes

C The influence of the distance between the electrodes, of their placement and size in bipolar surface recording

D The distribution of the electrical activity within a muscle during isometric contraction

PROCEDURE IN GENERAL

Muscles

The experiments were performed on the masseter the temporal and the brachial biceps muscles The brachial biceps muscle was included because the loads and therefore the degree of activity could be varied over a larger range in this muscle than in the temporal and masseter muscles

Electrodes

The intramuscular recordings were obtained by concentric needle electrodes In the case of the muscles of mastication the outer diameter of the cannula was 0 45 mm and the length 20 or 30 mm In the experiments on the brachial biceps muscle the diameter of the electrode was 0 65 mm and the length 42 mm For all electrodes the core was a platinum wire with a diameter of 150 μ and a bared tip of 0 07 mm^2 The needle electrodes were sterilized in boiling water for 20 minutes and before insertion the skin was cleaned with 70 per cent alcohol

Surface recording was carried out with electrodes made of tin Bipolar electrodes in a standard position refers to electrodes with a contact surface of 3 × 10 mm placed 10 mm apart along the fibres and with the long axis of the electrodes transverse to the direction of the fibres The electrodes used in unipolar" recording were circular with a diameter of 10 mm The skin was thoroughly cleaned with ether and the electrodes were covered with a paste*) which dissolved some of the epithelial horny layer and caused slight erythema The unipolar electrodes were fixed with cotton wool soaked in collodion The bipolar electrodes were placed on a piece of adhesive tape at the desired distance and thereafter fastened to the skin Over the temporal muscle the tape was sealed with collodion

The earth electrode (3 3 × 18 cm) was placed proximally to the recording electrodes when recording from the brachial biceps muscle and was wrapped around the neck when recording from the muscles of mastication

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*) Composition (1000 g) powder of troganth 15 g glycerin 45 g distilled water 500 g potassium bitartrate 7 g sodium chloride 189 g powder of pumice stone 242 g liquified phenol 2 g

Apparatus

Most previous studies of the electrical activity in the muscles of mastication were performed with electroencephalographs Therefore to have a basis of comparison some of the methodological investigations were made by recording with an eight channel electroencephalograph (KAISER type no E 1000) The input impedance was 2 MΩ in parallel with 2000 pF the frequency response was 3 db down at 1 5 and 115 cycle/sec The inkwriter recordings were measured graphically as described (*Møller* 1958)

Additional methodological experiments and all investigations of muscle activity during natural function were carried out by means of a three channel electromyograph with photographic recording from cathode ray oscilloscopes (DISA type no 13A50) The frequency response was 3 db down at 2 and 10 000 cycle/sec the input impedance was 100 MΩ in parallel with 60 pF To reduce artefacts from movements of electrodes and cables the lower limiting frequency was increased to about 20 cycle/sec

The mean voltage of the electromyograms was obtained by replacing one amplifier of the electromyograph by a dual beam mean voltage unit (DISA type no 13B05) in which the amplified action potentials are passed through a RLC filter after full wave rectification By this procedure two electromyograms and their corresponding mean voltages were displayed on three oscilloscopes and recorded on photographic paper (Fig 6) The two mean voltage tracings were identified by their different light intensities (thin trace and fat' trace)

The amplification of the electromyograms could be adjusted step-wise from 1 mm = 3 μV to 1 mm = 1000 μV the amplification of the mean voltage could be varied continuously from 1 to 5 times

In the *static experiments* (methodological studies postural activity full effort) the gain of the mean voltage was 5 times and adjustments were obtained by altering the amplification of the electromyograms The recording speed was 5 cm per sec

The mean voltage was measured on a section of the record corresponding to one second of recording (5 cm) An average line was drawn through the thin and the fat trace parallel to the base line The distances from these lines to the base line were measured with an accuracy of one millimetre (Fig 6) this equaled from 2 μV (postural activity high gain) to 20 μV (full effort low gain) Each experiment was performed twice and the average from the two recordings was used for calculation

In the *dynamic experiments* (mastication and swallowing) the onset and duration of the electrical activity were determined from the directly recorded electromyograms The sensitivity was kept constant at 20 μV per mm

high enough to show changes in the activity with sufficient accuracy The degree of activity during moderate and strong effort was determined from the mean voltage recording Adjustment of the gain of the mean voltage was necessary as the relatively high amplification caused overloading of the amplifiers of the electromyograph during maximal activity With three channels available it was impossible at the same time to record the electrical activity with a constant and high gain (uniform indication of measuring points) and with the gain adjusted to avoid overload at the highest degree of activity The recording speed in the dynamic experiments was 20 cm per sec

Calibration of the mean voltage unit was obtained from the calibration signals of the amplifiers of the electromyograph The calibration signals and the base line of the mean voltage were recorded at the end of each experiment.

Sources of error in the dynamic recordings were (1) the delay of the mean voltage in reaching maximal deflection and (2) the effect of over loading the amplifiers on the mean voltage

With the mean voltage unit (DISA, type no 13B05) there was a 55 msec delay to obtain maximal deflection when a train of diphasic rectangular pulses was put in (Fig 7 A) This delay could be disregarded in the static experiments when the recordings covered one to three seconds The influence of the delay when recording electrical activity during chewing with its brief strong contractions was estimated in the following way, trains of rectangular pulses and their mean voltages were recorded on the electromyograph The pulse duration was 3 msec the frequency 150 per sec and the amplitude was varied manually to simulate the increase and decrease in amplitude of the interference pattern during chewing Each train had a duration of about 300 msec as did the activity in the anterior temporal muscle during one chewing stroke The time course of the amplitude of the pulse train and its mean voltage were evaluated at intervals of 25 msec and plotted on a percentage scale (Fig 7 B and C) The mean voltage followed the changes in pulse amplitude with a delay of 15 msec

The effect of overloading the amplifiers of the electromyograph was evaluated by comparing the mean voltage recorded with and without overload The same electromyogram was recorded (1) with maximal amplification of the mean voltage (5 times) and with an amplification on the electromyograph which prevented it from being overloaded and (2) with a high amplification on the electromyograph (20 μV per mm) and with a gain on the mean voltage which prevented it from being overloaded

To vary the degree of overload widely recordings were made both with needle and with surface electrodes of maximal activity during chewing (Fig

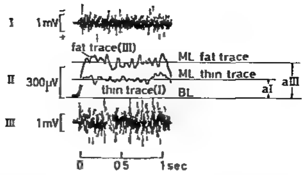

Fig 6

Simultaneous recording of two electromyograms (I and III) *and their mean voltages* (II)
In II the thin trace corresponds to the electromyogram in I the fat trace to that in III
BL Base line of the mean voltage
ML Measuring lines thin trace electromyogram I fat trace electromyogram III
aI and aIII Mean voltage indicating the degree of electrical activity in the static experiments (postural activity and full effort)

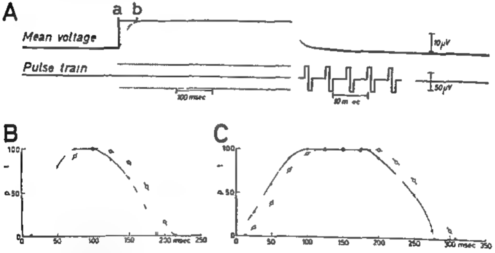

Fig 7

Delay in mean voltage recording

A Delay of mean voltage unit in reaching the true mean voltage for a train of diphasic rectangular pulses (right) Distance between *a* and *b* corresponds to 55 msec

B and C The amplitude (full lines) and the mean voltage (broken lines) of trains of rectangular pulses The pulse duration was 3 msec the frequency 150 per sec and the amplitude was varied manually to simulate the increase and decrease in amplitude of the interference pattern during chewing

Ordinate amplitude of the pulse train and its mean voltage in per cent of maximal deflection

Abscissa. time zero corresponds to the onset of the first pulse of the train

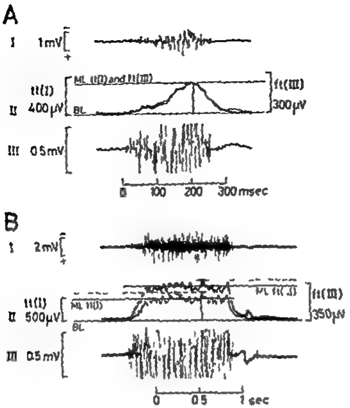

Fig 8

To illustrate the evaluation of the mean voltage in the right masseter muscle recorded simultaneously with and without overload of the amplifiers The recordings were obtained with a concentric needle electrode during chewing on the right side (A) and during maximal bite in the intercuspal position (B)

I and thin mean voltage trace (tt) of *II* undistorted recording with maximal gain of the mean voltage

III and fat mean voltage trace (ft) of *II* recording with overload and reduced gain of the mean voltage

The activity during chewing (A) was measured with respect to the maximal mean voltage (distance between ML and BL fat and thin trace coincide) Measurements of the recordings obtained during maximal bite (B) included the average level (distance between ML and BL) and the maximum (distance between broken line—above ML—and BL) of the mean voltage

Note difference in calibration of the thin (tt) and fat (ft) mean voltage traces

Subject M 33 male 27 years old

8 A) and of activity during maximal bite in the intercuspal position (Fig 8, B) The activity was recorded from the right masseter muscle of two subjects and the mean voltage was measured to the nearest millimetre corresponding to an accuracy of 15–40 μV depending on the degree of amplification

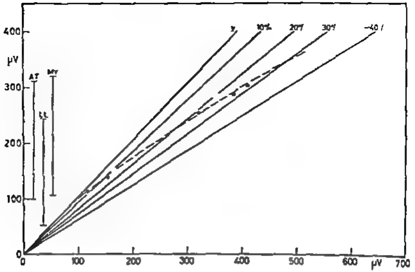

Fig 9

The mean voltage recorded with overload of the amplifiers as a function of the mean voltage obtained with undistorted recording Each point represents the average of 20 observations The full diagonal lines indicate the degree of reduction in mean voltage The broken line shows the average relation between distorted and undistorted recording the deviation from the line y = x indicating the degree of distortion The vertical lines (left) indicate the range of the maximal mean voltages during chewing and swallowing (AT anterior temporal muscles chewing MY mylohyoid muscles swallowing LL muscles of the lower lip chewing)

- ● subject M 10 male 37 years old
- ○ subject M 33 male 27 years old

In Fig 9 the mean voltage with overload (ordinate) and without (abscissa) are compared with the range in maximal mean voltages during chewing and swallowing The overload could have diminished the maximal mean voltages of the elevator and depressor muscles by no more than 30 per cent in the muscles of the lips the reduction could have been at most 20 per cent

Statistical analysis

The physical variables were studied by measuring their influence on the linear relation between electrical activity and isometric tension The mean voltage as a function of load was analysed by linear regression analysis) with the load as independent (x) and the mean voltage as dependent variable (y)

The individual regression line In all experiments the mean voltage (y) varied linearly as a function of load (x) A preliminary calculation of the variances demonstrated that

*) The principles of the statistical analysis used were taken from *Hald* (1952) Most of the statistical calculations were carried out on a computer

the variance of y increased with the load. A few experiments on the muscles of mastication revealed an almost constant variance over the entire range of loads because only a small fraction of the load acted on the muscle concerned. In the case of the brachial biceps muscle the smallest external load was zero and the function $s^2 (x + 1)$, where s is a constant, was used as the variance $V\{y/x\}$. The corresponding function for the temporal and masseter muscles was $s^2 (x + {}^1/_2)^2$. The applicability of these functions was tested by means of Bartlett's test; since the calculated values did not exceed the 97.5 per cent fractile of the χ^2 distribution except in one case, the existence of a common factor s was assumed and a combined estimate made. With the previously mentioned functions as the variance $V\{y/x\}$ it follows that the symbols $\bar{x}$ and $\bar{y}$ used below indicate the *weighted and not the arithmetic means of x and y*. With one exception the probability that the slope of the regression line could have arisen by chance (slope = 0) was less than five per cent.

Comparison of regression lines. The regression lines were compared pair-wise with regard to (1) slopes, (2) identity, (3) the mean voltages corresponding to a minimal external load, and (4) loads corresponding to mean voltage zero (brachial biceps muscle only).

The comparison at loads corresponding to mean voltage zero (4) was based on the assumption that the loads at this point did not differ significantly if the source of the electrical activity was the same. Because of the weight of the forearm the load on the muscle was not zero at zero external load and calculation of the loads corresponding to mean voltage zero, i.e. the intersection of the regression lines with the abscissa, necessitated extrapolation. In the brachial biceps muscle a comparison of the loads corresponding to $y = 0$ was acceptable since the extrapolation was small as compared to the range of loads applied. In the temporal and masseter muscles the small slope of the regression lines would have necessitated wide extrapolation, therefore these lines were compared instead at the minimal external load (0.5 kg).

The comparison with regard to slopes, identity and the mean voltages at minimal external load were made by means of the t test with $f = N_1 + N_2 - 4$ degrees of freedom, N_1 and N_2 being the number of observations for each line. As the variation about the regression lines in some cases differed significantly, the t test should have been modified with respect to f. However, considering the large number of observations contributing to each line (minimum of N: 88) this correction is of little importance; the t value for $f = 100$ was used.

The comparison at mean voltage zero was carried out by solving the regression equation with regard to the independent variable

$$x_0 = x - \frac{y}{b}$$

and comparing the calculated loads according to the following considerations*)
let $\eta = \alpha + \beta (x - \bar{x})$ be the theoretical and $y = a + b (x - \bar{x})$ the empirical regression equation; let ξ indicate the theoretical x value corresponding to $\eta = 0$; it follows that

$$z = a + b (\xi - x)$$

is normally distributed with a mean

$$m\{z\} = 0 \text{ and a variance } V\{z\} = S_{y,x}^2 + S_{(b)}^2 (\xi - \bar{x})$$

*) The test applied for the comparison of the regression lines at mean voltage zero was *suggested by E. Jensen, Regnecentralen A/S, Copenhagen.*

Therefore $t = \frac{m\{z\}}{s\{z\}}$ has a t-distribution with N − 2 degrees of freedom N being the number of observations contributing to the regression line

With y_1 and y as the two regression lines $t_1 = \frac{m\{z_1\}}{s\{z_1\}}$ was calculated after substitution of ξ with x_0 and then $t = \frac{m\{z\}}{s\{z\}}$ was calculated replacing ξ with x_{01}. A significant value of either t_1 or t implies rejection of the hypothesis of a common source of activity

Subjects

The methodological investigations were performed on 33 subjects 5 females and 28 males The subjects were 18–49 years of age and included staff members (8) students (14) and patients (11) from the Royal Dental College in Copenhagen The subjects were without signs or symptoms of neuromuscular disease or disorders of the temporomandibular joints and all had teeth in good or fairly good condition The type of occlusion was not considered

RESULTS

Impedance of the electrodes

Buchthal et al (1954) observed a substantial decrease in the impedance of the electrodes when the measuring voltage exceeded 100 mV In the present study impedances were measured of bipolar surface concentric needle and earth electrodes with a 50 μV signal at 50 500 and 5000 cycle/sec*) in the masseter temporal and brachial biceps muscles of the same subject

Earth electrodes The electrodes were 5 5 × 28 3 3 × 18 and 1 5 × 18 cm They were made of lead and covered with flannel moistened with a saturated solution of sodium chloride which was wrapped around the forearm or neck One flat surface of the electrode was entirely in contact with the skin except when the largest electrode was placed on the wrist The average impedances (earth to earth electrode) and their standard deviations are given in Table I The variation was greatest at 50 cycle/sec then the 1 5 × 18 cm electrode placed just above the wrist had the highest impedance With this exception impedances did not vary systematically with the size or position of the electrode

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*) I am indebted to A Rosenfalck EE, who kindly performed the measurements of the impedance of electrodes and of the rejection of common voltage (p 42)

Table 1

Numerical impedances (|Z|) between two earth electrodes wrapped around the forearm or neck measured with a 50 µV signal at 50 500 and 5000 cycle/sec One flat surface of the electrodes (5 5 × 28 3 3 × 18 and 1 5 × 18 cm) was entirely in contact with the skin except when the largest electrode was placed on the wrist (area of contact 110 cm²) Subject M 10 male 37 years old

Cycle/sec	|Z| Mean in kΩ	Standard deviation	Number of measurements
50	3 1	1 7	9
500	0 8	0 2	9
5000	0 2	0 06	9

Recording electrodes The impedances were measured of two bipolar surface electrodes (impedance lead to lead) in standard positions over the right and left brachial biceps masseter and temporal muscles The impedance core to cannula of the concentric needle electrode and the impedances to earth (5 5 × 28 cm electrode on the wrist) of the core cannula and each lead of a bipolar surface electrode were measured in or over the brachial biceps muscle (Table 2, B–D)

The impedance of the *surface electrodes* (1 and 2) depended on

1 Individual differences between the electrodes Electrode pair *2* (a, f i) had a higher impedance than *1* (b g, h)

2 The site Electrodes placed on the temporal muscle with *f* above the hair line and *g* at the hair line showed high impedances electrodes placed over the masseter muscle (h i) had the lowest impedances

3 Placement procedure Scrubbing the skin with ether so vigorously as to produce erythema (between b and c and between d and e) caused a 30 (b and c) to 70 (d and e) per cent lower impedance

The effect of the variation in impedance of the surface electrodes on the mean voltage could be diminished by using an amplifier with a high input impedance At 50 cycle/sec the impedance of the surface electrodes was 0 2 to 20 per cent of the input impedance of the electroencephalograph at 500 cycle/sec 1 to 30 and at 5000 cycle/sec 5 to 50 per cent. When recording with the electromyograph the impedance of the surface electrodes did not exceed 1 per cent of the input impedance at 50 cycle/sec and was below 3 per cent at 500 and 5000 cycle/sec

The impedances to earth of each of the two leads of a bipolar surface electrode differed by a factor of 2 5 at 50 cycle/sec the difference was less at 500 cycle/sec and negligible at 5000 cycle/sec (Table 2 C)

Table 2

Numerical input impedances (|Z|) of amplifiers and numerical impedances of recording electrodes[1] *measured with a 50 µV signal at 50 500 and 5000 cycle/sec* (subject M 10 male 37 years old)

Cycle/sec		|Z| in kΩ 50	500	5000
A Input impedances				
electromyograph (DISA type no 13A50)		27000	2000	320
electroencephalograph (KAISER type no E 1000)		1200	150	16
B Impedances of two pairs of surface electrodes (1 and 2 lead to lead)				
pair of electrodes	muscle			
2 (a)	left brach biceps	215	54	8
1 (b)		34	23	7
1 (c)		22	16	5
1 (d)	right brach biceps	28	18	5
1 (e)	"	8	7	3
2 (f)	right temporal	118	43	7
1 (g)	left temporal	[illegible]	37	8
1 (h)	right masseter	2	1 5	1
2 (i)	left masseter	11	9	3
C Impedances of a pair of surface electrodes (2 left brach biceps)				
lead to lead		203	50	7
each of the two leads to earth		[illegible]	21	3
		207	31	4
D Impedances of a concentric needle electrode (left brach biceps)				
core to cannula		95	32	7
core to earth		107	25	7
cannula to earth		2	0 7	0 4

[1] Leading off areas surface electrodes 30 mm concentric needle electrodes 0 07 mm² (core) and 50 mm (cannula) earth electrode 110 cm²

In *the concentric needle electrode* the impedances core to earth and core to cannula were almost identical the impedance of the core to earth was 50 times that of the cannula (Table 2 D)

The surface electrodes sometimes had higher impedances than the needle electrode (cf Fig 10) and the surface electrode cannot always be considered to represent a low impedance pick up

Table I

Numerical impedances (|Z|) between two earth electrodes wrapped around the forearm or neck measured with a 50 μV signal at 50 500 and 5000 cycle/sec One flat surface of the electrodes (5 5 × 28 3 3 × 18 and 1 5 × 18 cm) was entirely in contact with the skin except when the largest electrode was placed on the wrist (area of contact 110 cm²) Subject M 10 male 37 years old

Cycle/sec	|Z| Mean in kΩ	Standard deviation	Number of measurements
50	3 1	1 7	9
500	0 8	0 2	9
5000	0 2	0 06	9

Recording electrodes The impedances were measured of two bipolar surface electrodes (impedance lead to lead) in standard positions over the right and left brachial biceps masseter and temporal muscles The impedance core to cannula of the concentric needle electrode and the impedances to earth (5 5 × 28 cm electrode on the wrist) of the core cannula and each lead of a bipolar surface electrode were measured in or over the brachial biceps muscle (Table 2, B–D)

The impedance of the *surface electrodes* (1 and 2) depended on

1 Individual differences between the electrodes Electrode pair *2* (a f i) had a higher impedance than *1* (b g h)

2 The site Electrodes placed on the temporal muscle with *f* above the hair line and *g* at the hair line showed high impedances electrodes placed over the masseter muscle (h i) had the lowest impedances

3 Placement procedure Scrubbing the skin with ether so vigorously as to produce erythema (between b and c and between d and e) caused a 30 (b and c) to 70 (d and e) per cent lower impedance

The effect of the variation in impedance of the surface electrodes on the mean voltage could be diminished by using an amplifier with a high input impedance At 50 cycle/sec the impedance of the surface electrodes was 0 2 to 20 per cent of the input impedance of the electroencephalograph, at 500 cycle/sec 1 to 30 and at 5000 cycle/sec 5 to 50 per cent When recording with the electromyograph the impedance of the surface electrodes did not exceed 1 per cent of the input impedance at 50 cycle/sec and was below 3 per cent at 500 and 5000 cycle/sec

The impedances to earth of each of the two leads of a bipolar surface electrode differed by a factor of 2 5 at 50 cycle/sec the difference was less at 500 cycle/sec and negligible at 5000 cycle/sec (Table 2 C)

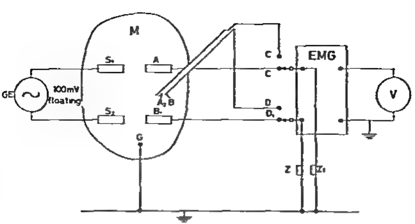


Fig 11

Rejection of common voltage Diagram of the set up to determine the in phase gain of the recording system (tissue electrodes and electromyograph)

GE	sine wave generator (Hewlett Packard type no 200 CD) connected to the power line by a double shielded transformer to float the output (Guld 1961)
EMG	difference amplifier (electromyograph, DISA, type no 13A50)
S1–S2	surface electrodes to which the signal was applied
A1–B1	leads of surface electrode for recording connected to the input of the difference amplifier at C1 and D1
A2–B2	leads of concentric needle electrode for recording (A2 core, B2 cannula) connected to the input of the difference amplifier at C2 and D2
Z_1	input impedance of the electromyograph the experiments were carried out with $Z_1 = 100$ MΩ in parallel with 60 pF and 1 8 MΩ in parallel with 60 pF
V	vacuum tube voltmeter measuring V_{RM} (Brüel and Kjær type no 2409)
M	brachial biceps muscle
G	earth electrode

high with surface electrodes (50 between 5 and 100 cycle/sec 100 above 500 cycle/sec) With an input impedance of 100 MΩ in parallel with 60 pF the rejection ratio was 2000 at 5 cycle/sec 400 to 700 between 10 and 100 cycle/sec and 200 at 500 cycle/sec Between 1000 and 10 000 cycle/sec the rejection ratio with surface recording was 150 for the high and low input impedances

With concentric needle electrodes the rejection ratio with an input impedance of 100 MΩ in parallel with 60 pF was 10 times greater than with an input impedance of 1 8 MΩ in parallel with 60 pF up to about 100 cycle/sec This difference diminished with increasing frequency and disappeared at about 1000 cycle/sec

The results illustrate the relationship between input impedance and common voltage rejection Increasing asymmetry in the impedance of electrodes diminished the rejection ratio as exemplified by the difference between the rejection ratios obtained with surface and needle electrodes

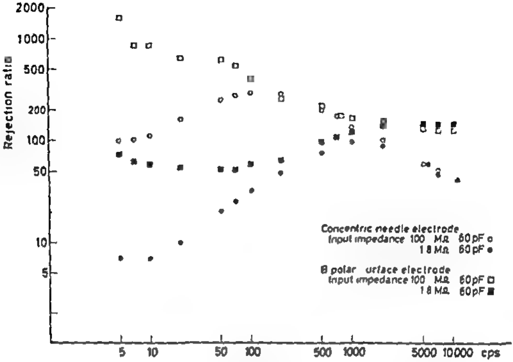


Fig. 12

Rejection of common voltage in recordings with surface and concentric needle electrodes with an input impedance of the amplifier of 100 MΩ in parallel with 60 pF and 1 8 MΩ in parallel with 60 pF (subject M10 male 37 years old)

Ordinate rejection ratio defined as the ratio between the out of phase and in phase gain of the recording system (tissue electrodes and electromyograph) logarithmic scale

Abscissa measuring frequency logarithmic scale

Comparison of the electromyograph and the electroencephalograph

In ten subjects the mean voltage was measured during isometric contraction First the electromyogram was recorded on an inkwriter (electroencephalograph) and the mean voltage was measured graphically from the height of the envelope of the interference pattern (*Møller* 1958) then the electromyogram was photographed (electromyograph) and the mean voltage was measured graphically as described above and electronically (see p 33)

Loads were applied to the right brachial biceps muscle via a hand grip During the recording the arm was flexed 90° at the elbow and the upper arm hang vertically The first recording was made with the arm unloaded Thereafter loads were applied from 1 to 10 kg adding 1 kg at a time The electrical activity was picked up simultaneously from a pair of bipolar surface electrodes in a standard position (see p 32) and from a concentric needle electrode

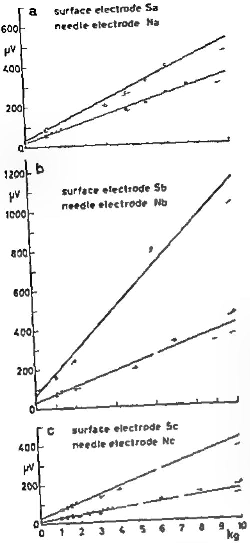


Fig 13
The mean voltage of the electromyogram recorded above and within the brachial biceps muscle as a function of the load on the flexors of the upper arm (average from 10 subjects)

Inkwriter recordings (electroencephalograph) measured graphically (a) and recordings with cathode ray oscillograph (electromyograph) measured graphically (b) and electronically (c mean voltage unit) The full lines (regression lines) were determined by regression analysis the stipled lines indicate the uncertainty (±2SE) of the regression lines

In all experiments the mean voltage tended to increase more than linearly with the load (Fig 13 a b and c) Since increasing loads were applied and the intervals of rest were short (about 30 sec) this tendency was probably due to fatigue Since the variations about the regression lines were similar, and since the variation was well within the limit of certainty of linearity the results were compared by regression analysis

From the electroencephalographic records measured graphically (Sa and Na Fig 13 a Table 3) the slope of the relation between mean voltage and load was less steep when surface electrodes than when needle electrodes were used In the unloaded condition there was no difference whether surface or needle electrodes were used

Table 3

Comparison of the mean voltage (brachial biceps muscle) *obtained with bipolar surface* (Sa Sb Sc) *and concentric needle* (Na Nb Nc) *electrodes* (cf Fig 13) The mean voltages as linear functions of load were compared with respect to slope (slope-differences) identity (y-differences) mean voltage at minimal external load ($y_{(x\ min)}$-differences) and load at mean voltage zero (x_o-differences) The t test (t) was applied 10 subjects average of two recordings on each

Electrodes(1) compared	t slope differences	t y differences	t $y_{(x\ min)}$ differences	t x_o differences
Sa/Na	4.1**	3.9***	1.6	Sa/Na 0.27 Na/Sa 0.21
Sb/Nb	9.8 *	7.9 *	2.5*	Sb/Nb 1.22 Nb/Sb 1.54
Sa/Sb	1.5	2.8*	1.8	Sa/Sb 1.55 Sb/Sa 1.41
Na/Nb	8.1***	11.8 *	2.0	Na/Nb 0.14 Nb/Na 0.20
Sc/Nc	10.5***	9.0***	3.2	Sc/Nc 0.86 Nc/Sc 1.15

(1) Sa and Na inkwriter (electroencephalograph) recordings measured graphically
Sb and Nb cathode ray oscilloscope (electromyograph) recordings measured graphically
Sc and Nc same recordings as Sb and Nb but measured electronically (mean voltage unit)

t* $0.01 < p < 0.05$ t** $0.001 < p < 0.01$ t*** $p < 0.001$

From the electromyographic recordings measured graphically (Sb and Nb Fig. 13 b Table 3) a much steeper slope of the relation between mean voltage and load was derived with intramuscular than with surface recordings The difference in mean voltage was significant even at minimal load the extrapolated regression lines coincided at mean voltage zero

The electronically measured mean voltages of the electromyographic recordings (Sc and Nc, Fig 13 c Table 3) showed about the same relationship between surface and needle recordings as did the graphically determined mean voltage it was about three times greater when the recordings were obtained with needle than with surface electrodes

In summary the mean voltages measured graphically from the electromyographic and electroencephalographic records indicated that results obtained with surface and with needle electrodes were affected differently by the different recording systems The slopes of the relation between mean voltage and load were identical when surface electrodes were used whether

the electroencephalograph or the electromyograph was used for recording (Sa and Sb Table 3) but the mean voltage recorded with the electroencephalograph was about 25 per cent lower With the smallest load the differen ce was insignificant When concentric needle electrodes were used (Na and Nb Table 3) *the mean voltage recorded with the electroencephalograph* and the electromyograph differed significantly The mean voltage recorded with the electroencephalograph averaging 60 per cent less the slope of the re gression lines was different but they coincided at mean voltage zero

Surface recording and recording with concentric needle electrodes

The effect of electrode positioning was studied in two series of experiments In the first series unipolar surface recordings were obtained from the right anterior and posterior part of the temporal and from the masseter muscle The indifferent electrode was placed on the vertex of the skull and in the case of the *anterior temporal and masseter muscles* on the earlobe as well In the second series of experiments the bipolar recordings were with surface *electrodes in a standard position over the same muscles Both series* included simultaneous recordings with concentric needle electrodes inserted immedi ately beneath the surface leads (Fig 14, a and b)

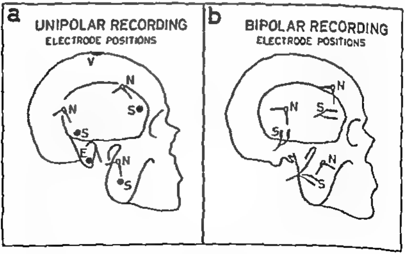


Fig 14

Positions of electrodes in the experiments with unipolar and bipolar surface recording (S) *and recording with concentric needle electrodes* (N) During unipolar recording (a) the indifferent electrode was placed on the vertex of the skull (V) or on the earl

and during horizontal loading of the anterior part (Table 4 N1 and N2 ȳ-differences) *there was no difference in the slope of the relation between* mean voltage and load nor at minimal load (y_{min}-differences)

In all experiments the mean voltage obtained with bipolar surface (SB) and simultaneously with intramuscular electrodes (N2) showed the same relation to load and were identical at minimal load The average mean voltage was also the same except during vertical loading of the anterior temporal muscle (Fig. 15 A a Table 4 a) when the average mean voltage was slightly higher with bipolar surface recording. In view of the paucity of activity the similarity between bipolar surface and intramuscular recordings was consistent with the electroencephalographic recordings from the brachial biceps muscle

When the smallest load was applied unipolar surface recordings (SV SE) gave significantly higher mean voltages than did concentric needle electrodes (N1) This applied to recordings obtained from the masseter muscle during vertically (Fig 15 A c_1 Table 4 c) and horizontally applied loads (Fig 15 B c_1 Table 4 c) and from the anterior part of the temporal muscle during horizontally applied loads (Fig 15 B a_1 Table 4 a)

The slight activity found with bipolar surface and concentric needle electrodes in the masseter muscle loaded vertically and horizontally and in the anterior temporal muscle subjected to horizontal loads is in keeping with the findings of *Carlsoo* (1952) Therefore the higher mean voltage obtained with unipolar surface electrodes must be due to action potentials from the anterior part of the temporal muscle when the pull was vertical and from the posterior part of this muscle when the pull was horizontal

Bipolar surface recordings

The influence on the mean voltage of the distance between surface electrodes of their placement and of their size was studied in experiments carried out on the brachial biceps muscle Application of the loads has been described (p 44) the inkwriter (electroencephalograph) recordings were measured graphically

The distance between the 3 × 10 mm electrodes was varied in two experiments experiment I was carried out at a load of 2 kg One pair of electrodes was 10 mm apart and the distance between another pair was increased from 2 5 mm to 40 mm and thereafter diminished again to 2 5 mm Hence two sets of recordings were obtained from each of five subjects In experiment II recording was from electrode pairs at fixed distances from each

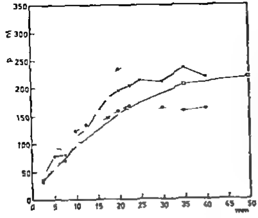


Fig 16

The mean voltage in the brachial biceps muscle obtained with surface electrodes with different distances between the leads in per cent of the mean voltage obtained with 10 mm between the leads as a function of the electrode distance The recordings were obtained at a load of 2 kg and the electrodes placed along the fibres the inkwriter (electroencephalograph) recordings were measured graphically (5 subjects average of 2 recordings on each)

X———X mean voltage in per cent
●- - - -● ± SD (standard deviation)
□———□ data at a load of 2 kg taken from Fig. 17

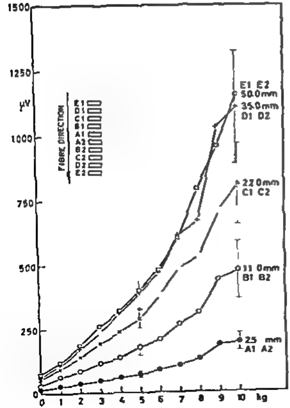


Fig 17

The mean voltage in the brachial biceps muscle obtained from pairs of surface electrodes placed at fixed distances along the fibres as a function of load The inkwriter electroencephalograph) recordings were measured graphically (5 subjects average of two recordings on each) The uncertainty (± SE) is indicated at 5 and 10 kg at electrode distances of 35 and 50 mm only the uncertainty at 35 mm is indicated

other (shown in Fig 17) and the loads were increased in 1 kg steps from 1–10 kg In the five subjects the recordings were repeated with the same successive loads

In experiment I the mean voltage from the two leads at different distances from each other was expressed in per cent of the mean voltage recorded from the electrodes 10 mm apart and was plotted as a function of the distance between the electrodes (Fig 16) The mean voltage increased with the distance between electrodes up to 25–30 mm and was constant at larger distances This result was confirmed by the results from experiment II where the mean voltage was plotted as a function of load (Fig 17) the curves corresponding to distances of 2 5 11 22 and 35 mm showed an increment in mean voltage a further increase in distance to 50 mm showed no additional increase in mean voltage

The agreement between the results from the two series of experiments is illustrated in Fig 16 where the mean voltages from experiment I are plotted as well as the mean voltages obtained in experiment II at a load of 2 kg

The influence of the placement of the electrodes on the mean voltage was studied by recording simultaneously from two pairs of surface electrodes one with the leads placed parallel with the fibres the other with the leads placed transversely to the long axis of the fibres the leads were 15 mm apart (Fig 18) These experiments were performed on 10 subjects with loads from 1 to 10 kg and the results were compared by regression analysis

The mean voltage was 35 per cent less ($p < 0\ 01$) when leading off transversely to the fibre direction than when recording along the muscle fibres (Fig 18)

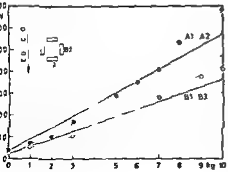


Fig 18

The mean voltage in the brachial biceps muscle obtained with surface electrodes placed along the fibres (A1–A2) *and transversely to the direction of the fibres* (B1–B2) *as a function of load* The electrodes were placed 15 mm apart and the inkwriter (electroencephalograph) recordings were measured graphically (10 subjects average of 2 recordings on each) The full lines (regression lines) were determined by regression analysis the stipled lines indicate the uncertainty (± 2SE) of the regression lines The slope of the line A1–A2 was significantly larger than that of line B1–B2 ($t = 3\ 2$ $0\ 001 < p < 0\ 01$)

Table 5

The mean voltage in the brachial biceps muscle as a function of the si e of the electrode The mean voltage was expressed in per cent of the mean voltage obtained with surface leads of 10×3 mm placed along the fibres (a) and that obtained with a concentric needle electrode (b) The recordings were obtained with a load of 2 kg the distance between surface leads was 10 mm the inkwriter (electroencephalograph) recordings were measured graphically (two recordings on each of 5 subjects)

Extent of electrodes transversely to the fibre direction (mm)	Extent of electrodes along the fibres (mm)				
	1 5	3 0	5 0	7 5	10 0
a					
5	50	50	68		
10	50	56	65	74	[illegible]
15		54	58		
20		55	67		
b					
5	87	94	108		
10	74	91	103	119	126
15		95	112		
20		96	105		

The influence of the size of the electrodes was studied in five subjects with a load of 2 kg Simultaneous recordings were obtained from electrodes of different sizes from a standard electrode (3 × 10 mm) and from a concen tric needle electrode The mean voltage recorded from the variable lead pair was expressed in per cent of that from the standard electrode and from the concentric needle electrode (Table 5 a and b) the mean voltage was greater when the larger area of contact was in the direction of the fibres

Distribution of activity within the muscle

The distribution of the electrical activity over the cross section of the brachial biceps muscle was studied in one subject at a constant load of 4 kg (about 20 per cent of the maximal force) The electrical activity was picked up by a multi electrode containing 14 leads with 2 5 mm between the centres of the leads (*Buchthal* et al 1957 a) The electrode was inserted transversely to the fibre axis With the cannula as indifferent electrode lead [illegible] was con nected to channel III of the electromyograph and the activity from the other leads was recorded successively on channel I The mean voltage recorded by each lead was measured electronically and was expressed in per cent of the mean voltage led off by lead 6 The activity varied by a factor of two even between sites very close to each other (Fig 19 a and b)

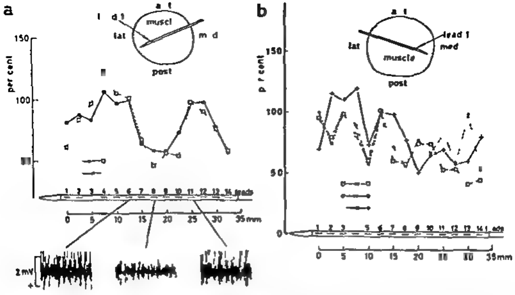
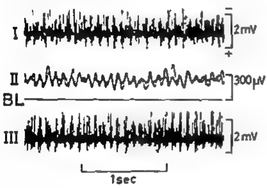


Fig 19

The mean voltage in the brachial biceps muscle recorded between the single leads and the cannula of a multilead electrode in per cent of the mean voltage at lead 6 The recordings were obtained at a load of 4 kg with two angles of insertion (a and b) The variation in the degree of activity is exemplified in *a* (below) The arrows indicate the sequence of the two series of recordings in *a* and the 3 series in *b* (subject M 10 male 37 years old)



Fg 20

Synchronous grouping of action potentials in the brachial b ceps muscle at the onset of fatigue Electromyograms (I III) and mean voltage (II) recorded on two leads (12 5 mm apart) of a multilead electrode during a load of 4 kg (cf Fig 19)

I and thin mean voltage trace of *II* lead 1 to cannula

III and fat mean voltage trace of *II* lead 6 to cannula

BL base line of the mean voltage

Subject M 10 male 37 years old

In these experiments synchronous grouping of action potentials (*Piper* 1907 *Buchthal* and *Madsen* 1950 *Lippold* et al 1957 and *Taylor* 1962) was observed at the onset of fatigue (Fig 20) the mean voltage tracings too, showed a 10–11 per sec rhythm Another effect of fatigue was an increase in average mean voltage from the first to the second experiment (Fig 19 lead 6 to cannula in *a* 150±4 µV SD = 21 µV, in *b* 231±8 µV SD = 52 µV)

DISCUSSION

Electrodes

The choice of leading off electrodes had to be adjusted to the small size of the muscles of mastication and to the fact that they are situated close to each other in addition some of them are difficult of access Moreover the subjects had to be able to chew and swallow with little or no interference from the electrodes

The external and internal pterygoid the digastric and the mylohyoid muscles could only be reached by means of needle electrodes Concentric needle electrodes were used and interference with chewing and swallowing was reduced by inserting one or two electrodes at a time

To obtain recordings without interfering with function the activity in the temporal the masseter and the lip muscles was led off by surface electrodes

A comparison of bipolar and unipolar surface recordings from the temporal and masseter muscles showed that ' unipolar ' placement recorded activity from that muscle which was most active during the experiment When leading off with a concentric needle electrode from the superficial portion of the masseter muscles during a horizontal pull of 1 5 kg *Carlsoo* (1952) found activity in only 2 of 40 experiments on 7 subjects I have confirmed this finding using bipolar surface electrodes and concentric needle electrodes Yet at the minimal load (0 5 kg) the unipolar surface electrodes recorded activity most likely picked up from the posterior temporal muscle

The voltage picked up by the indifferent electrode has been considered the main source of error with unipolar surface recording Several attempts have been made to reduce this error leads on the interconnected earlobes were used as indifferent electrode (*Perry* 1955) the indifferent electrode was placed on the back of the neck (*Greenfield* and *Wyke* 1956) or connected to earth (*Gopfert* 1952 *Gopfert* and *Gopfert* 1954) or a five point reference electrode was used (*Hickey* et al 1958) The error with unipolar surface recording arises from the placement of one of the electrodes at some distance from the muscle under study whereby activity conducted from other muscles does not appear as a common voltage This error is diminished by using bipolar surface electrodes with a distance between the electrodes which is small as compared to the size of the muscle activity conducted from adjacent muscles is nearly identical on the two leads and therefore rejected The distance between the electrodes their placement and size were kept constant since the mean voltage varies with these magnitudes The electrodes were placed 10 mm apart along the main fibre direction and the same type of electrode was used throughout The 10 mm distance was chosen because it recorded sufficient activity and yet permitted fix

ation of both leads within the limits of the muscles concerned To prevent part of the lead from lying outside the muscle the width of the electrode was 3 mm along the fibre axis and to facilitate fixation its length was 10 mm transverse to the direction of the fibres In the case of the muscles of the lips the area available for fixation made it necessary to use an electrode 5×3 mm

The impedance of surface electrodes has not previously been measured It varied widely (by a factor of 100 at 50 cycle/sec) depending on individual differences between the electrodes on their location and the placement procedure Electrodes placed on the temporal muscle had impedances similar to or even higher than those of concentric needle electrodes (*Buchthal* et al 1954 cf Table 2 and Fig 10), electrodes placed on the masseter muscles had impedances similar to those measured between earth electrodes ($1\ k\Omega < |Z| < 2k\Omega$ at 50 500 and 5000 cycle/sec) Finally the impedance of surface electrodes was reduced by as much as 70 per cent by repeated rubbing of the skin with ether

Measurements of the resistance between surface electrodes varied widely (*Greenfield* and *Wyke* 1956 and *Findlay* and *Kilpatrick* 1960 always below 50kΩ usually below 20kΩ *Kawamura* 1957 b less than 30kΩ *Kydd* 1959 less than 10 kΩ *Latif* 1957 and *Liebman* and *Cosenza* 1960 well below 3kΩ) In these measurements the capacitive component of the impedance was disregarded This component is important just in the frequency range of surface electromyograms (50–500 cycle/sec) while it is negligible above 5000 cycle/sec Therefore only impedances at 5000 cycle/sec ($1k\Omega < |Z| < 8k\Omega$ Table 2 Fig 10) are comparable to previous measurements of resistance (3kΩ to 50kΩ)

Amplification and recording

The degree of interference from potentials from neighbouring muscles depends on the input impedance of the amplifier and on the impedance of the electrodes The degree of distortion of amplitude depends both on the input impedance (voltage division) and on the upper limiting frequency of the recording system (amplification of spike components)

Rejection of common voltage With bipolar surface electrodes the rejection of common voltage was high even when the input impedance of the amplifier was low (1 8 MΩ in parallel with 60 pF) With concentric needle electrodes the asymmetry in impedance of the cannula and the core required a high input impedance when low frequency components (50 cycle/sec or less) were to be rejected This explains the large movement artefacts in re

cordings with needle electrodes and with amplifiers whose input impedance was low (*Moyers* 1949 and 1964 *Ekholm* and *Sirila* 1960 *Woelfel* et al 1960 *Ahlgren* and *Posselt* 1963)

Distortion of the amplitude When an electroencephalograph was used for recording (input impedance [illegible] MΩ in parallel with 2000 pF frequency range 1 5–115 cycle/sec) the mean voltage was 25 per cent (surface electrodes) and 60 per cent (needle electrodes) lower than when recording with an electromyograph (input impedance 100 MΩ in parallel with 60 pF frequency range 20–10 000 cycle/sec) This reduction was due to the low input impedance and the low upper frequency limit of the electroencephalograph At 50 cycle/sec the impedance of the electrodes varied between [illegible]2 and 20 per cent of the input impedance of the electroencephalograph at 500 cycle/sec between 1 and 30 per cent. Hence the smaller input impedance of the electroencephalograph can account for 10–15 per cent of the reduction in mean voltage with both surface and needle electrodes The additional decrease must be due to the smaller frequency range of the electroencephalograph In recordings with surface electrodes the high frequency components disappear because of the large distance between the source and the electrodes (*Buchthal* et al 1957 a *Hakansson* 1957) This explains the small reduction in mean voltage when the upper frequency limit is 115 cycle/sec (electroencephalograph) Due to the shorter distance between source and electrodes the recordings with needle electrodes contain more high frequency components Therefore these recordings benefit from the wide frequency response of the electromyograph and a significantly higher mean voltage was obtained than when recording with an inkwriter

Thus when recording with an inkwriter of the type used in most electroencephalographs it is impossible to avoid interference from neighbouring muscles and reduction of the amplitude when using needle electrodes for recording These recordings require the high input impedance and the wide frequency range provided by the electromyograph the recording instrument in the study reported here With an adequate amplification and recording system leading off with needle electrodes gave a three times larger mean voltage than simultaneous surface recordings

Distribution of activity

One factor which adds to the variation in mean voltage is exemplified by the uneven distribution of electrical activity observed in the brachial biceps muscle during a constant load The degree of activity could differ by a factor of two between sites of recording so close as to correspond to the un-

avoidable small shifts in the position of a needle electrode during natural function of the muscles of mastication A similar problem exists with surface electrodes (*Liebman* and *Cosenza* 1961, temporal muscle, *Sutton* 1962 brachial biceps muscle) This source of variation may be reduced by position ing the electrode carefully and by measuring repeatedly with different positions of the electrodes The uneven distribution of electrical activity during constant loading is a physiological variable which must be taken into account when a difference in mean voltage is interpreted as representing a difference in muscle function

Chapter III

ELECTRICAL ACTIVITY IN THE MUSCLES OF MASTICATION DURING NATURAL FUNCTION

Muscle coordination was studied by electromyography during chewing and swallowing at rest (postural activity) and during full effort The variation in electrical activity in the same subject and from subject to subject is described in statistical terms

METHODS AND MATERIAL

Mastication

The activity associated with mastication was studied in two experimental situations

I *Chewing of apple and white bread* which involves a reduction in size and a change in consistency of the bolus The way the bite was taken the size of the bite and the manner of chewing were left to the subject The recordings comprised the activity in the resting position during opening of the mouth during the bite and the first 5–6 chewing strokes Recording was then suspended but—during chewing of the apple—it was resumed for the last two or three strokes and during swallowing. Before recording the sequence of mastication was performed once to adjust the amplification and the position of the needle electrodes such that they did not cause discomfort The firm juicy apples were cut in quarters and chewed unpeeled The white bread was of the conventional Danish type soft with a relatively hard crust it was given in one cm thick slices spread with butter

II *Unilateral chewing* of chewing gum with unchanged size and consistency of the bolus Mastication was deliberately one sided to study the relation between " balancing side" chewing side and a preferred side of chewing and the activity in the muscles during chewing To ensure that chewing was confined to one side the bolus was kept small and cohesive The chewing

gum without candy cover was given in pieces of about 0 4 cm^3 It was chewed for 2–3 minutes before the experiment to obtain a uniform consistency and to ascertain that the subjects were able to perform a series of unilateral strokes In each experiment 4–5 chewing strokes were recorded

Swallowing

Two series of experiments were performed to study the activity associated with swallowing (1) swallowing to drain the saliva from the oral cavity, (2) swallowing in connection with mastication as a means of transportation in the digestive process Swallowing associated with drinking was not studied

When swallowing saliva the subject was instructed to indicate by a light when the necessary amount of saliva was present and to keep the mandible at rest until the recording had started Recording was continued until the activity had returned to the resting level Thus each experiment comprised 3–4 swallows preceded and succeeded by a period of rest The activity during swallowing of food was recorded in immediate succession to the activity during chewing of the apple The subjects were asked not to hasten swallowing Nonetheless conditions were not precisely as under natural circumstances and the increased activity with the mandible at rest which in some cases preceded and succeeded swallowing may be ascribed to the unfamiliar situation

Postural activity and full effort

Postural activity The activity in the muscles of mastication was studied with the mandible at rest and in the position corresponding to the earliest contact between the upper and lower teeth during natural closure ('occlusal position') The aim was to decide whether postural activity in any of the muscles concerned differed consistently in the two positions

Full effort The degree of electrical activity associated with full effort was recorded during

(1) maximal bite in the intercuspal position
(2) maximal bite on the incisors (incisive bite) and
(3) maximal protrusion and depression of the mandible

Recording of tooth contact

The study of mastication and swallowing included the recording of tooth contact. The make and the break of tooth contact were signalled by an elec

trical contact between two stainless steel bands (made of strips 8 × 0 08 or 5 × 0 08 mm) mounted on an upper and on a lower incisor (Fig 21 a and b) The band on the lower incisor was placed around the cervical third of the crown and a strip welded labially was passed over the incisal edge and welded orally The upper band was placed such as to make contact with the lower in the intercuspal position and in small lateral and protrusive movements The steel bands on the incisors did not signal tooth contact in positions posterior to the intercuspal position The bands were fitted tightly so they would stay without being cemented and the leads were fixed between the band and the tooth surface

Sufficient space for both bands (0 16 mm) was readily found consistent with the fact that contact between the upper and lower teeth in the intercuspal position is less pronounced in the incisor area than in the side regions (*Tryde* 1964 *Beyron* 1964) In subjects whose incisors did not touch in the intercuspal position (3 of 36) the bands were placed on the canines of the left or the right side

The leads from the bands were connected to a pulse generator (Fig 21 c) signalling the make and break of direct contact. These signals were easily distinguished from signals caused by electrolytic contact by their larger amplitude (Fig 21 d e and f) Whenever there were full crowns or cusp cover-

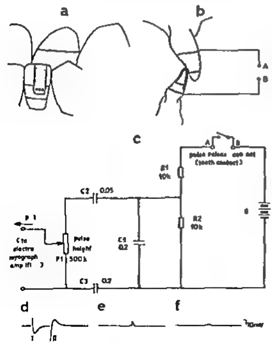


Fig 21
Recording of tooth contact

a Oral view of contact. stainless steel bands attached to an upper and lower incisor

b Sagittal view of contact bands A and B are terminals to pulse generator

c Pulse generator (Raun Electronic) A and B see b Make of tooth contact (direct contact between bands) charged the capacitors C1 C2 and C3 and caused a discharge adjustable in amplitude by the potentiometer P1 At the break of tooth contact the discharge was reversed

d Signals evoked at the make (I) and break (II) of direct contact between bands

e and f Signals obtained with electrolytic contact between the bands i e by placing the bands in saliva (e) or in a piece of apple (f)

ing inlays on the opposite teeth in the side regions tooth contact was determined there as well the contact leads were fixed to a steel ligature twisted around the cervical part of the teeth in question

Apparatus and electrodes

The electrical activity was recorded by a three channel electromyograph (DISA type no 13A50 and MGA) one of the amplifiers being replaced by a double beam mean voltage unit (DISA type no 13B05) This set up allowed simultaneous recording of two electromyograms and of their mean voltages (see Fig 6)

The bipolar surface electrodes were placed with their long axis transverse to the main direction of the fibres with an interelectrode distance of 10 mm along the fibre axis The size of the electrodes was 10 × 3 mm except on the lips where it was 5 × 3 mm The concentric needle electrodes had a length of 20 or 30 mm and a diameter of 0 45 mm In the case of the internal pterygoid muscles it was sometimes necessary to use an electrode which was 42 mm long and had an outer diameter of 0 65 mm (for details with respect to the positions of the electrodes see p 16)

Experimental procedure

To study the coordination in time of the various muscles during chewing and swallowing with a two-channel recording system (2 electromyograms and their mean voltages) the activity in the different muscles were compared with that of one muscle kept in constant connection with one of the recording channels Preliminary studies had indicated that the activity in the anterior part of the temporal muscle had a well defined pattern and the right anterior temporal muscle was chosen as reference (termed reference muscle) The activity in the other muscles (termed test muscles) and the signals of tooth contact were recorded successively on the other channel by means of a switch box During swallowing the recordings from the external and internal pterygoid digastric and left mylohyoid muscles were repeated with the right mylohyoid as reference muscle

The experiments (summarized in Table 6) were generally carried out in three to five sessions in a few subjects in two sessions The interval of time between subsequent sessions was more than three days in most subjects several months

Position of subjects The subjects were seated upright with the head unsupported on a couch with an adjustable back rest (Fig 22) The couch allowed the subject to recline at once if he felt faint but this occurred only twice The insertion of the needle electrodes was carried out under light nitrous oxide analgesia with the subject lying down For final adjustments and replacements of the electrodes the subjects sat in the recording position without analgesia

Table 6

Survey of experiments

Experiments			Muscles and number of subjects in each experiment																	Number of test muscles in each experiment (number of repetitions on each subject)
			Ant temp		Post temp		Mass		Int pteryg		Ext pteryg		Di gastric		Mylo hyoid		Lips		Tooth cont	
Main groups	Sub groups	Single series	r	l	r	l	r	l	r	l	r	l	r	l	r	l	upper	lower	Incisor region	
Chewing	Natural	Apple	Rf	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	16
		Bread	Rf	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	16
	Unilateral	r sided	Rf	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	16
		l sided	Rf	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	16
Swallowing	Saliva	Reference r ant temp m	Rf	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	16
		Reference r mylohyoid m							x	x	x	x	x	x	Rf	x				7
	Apple	Reference r ant temp m	Rf	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	16
		Reference r mylohyoid m							x	x	x	x	x	x	Rf	x				7
Posture and full effort	Postural ne	Rest pos	Rf	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		15
		Occlusal pos	Rf	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		15
	Full effort	Intercuspal pos	Rf	x	x	x	x	x	x	x	20	20	17	17						11
		Incisive bite	Rf	14	14	14	14	14	9	9	7	7	9	9						11
		Protrusion	Rf								35	35	29	29						4
		Opening	Rf								35	35	29	29						4

r right l left Rf reference muscle x experiment carried out on 36 subjects with less than 36 subjects the number is indicated

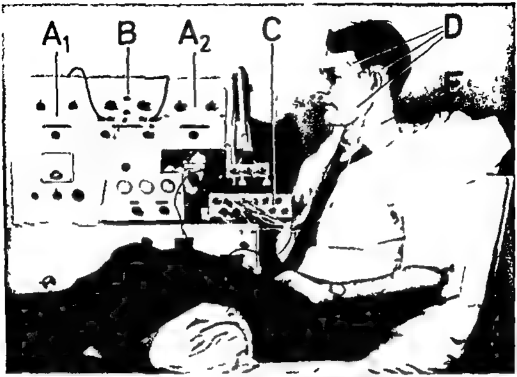


Fig 22

Experimental set up

A1 and A2 amplifiers of the electromyograph
B mean voltage unit
C switch box for electrodes (Raun Electronic)
D surface electrodes above the anterior and posterior part of the temporal and the masseter muscles
E earth electrode

Quantitative evaluation

The mean voltage recordings from the *static experiments* were evaluated as described (cf Fig 6) all data subjected to further calculations were the average of two observations

Chewing The onset and the cessation of activity was determined from the direct recordings of the electrical activity The duration of the highest degree of activity was determined from the mean voltage tracings measured from the time when the mean voltage reached 50 per cent of its maximum to the time when it had declined to 50 per cent In addition the activity was characterized by the maximal mean voltage and by the time of occurrence of this maximum The measurements of time were referred to the onset of the

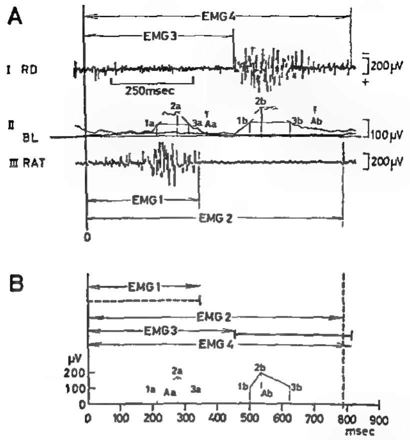


Fig 23a

To illustrate the evaluation of the activity in the reference and the test muscle (primary action) *during a single chewing cycle* (A) *and the diagrammatic presentation of time and amplitude data* (B)

Reference muscle (right anterior temporal RAT)

A *III* and fat mean voltage trace of *II* B stipled lines EMG 1 total duration of activity EMG 2 total duration of chewing cycle i e time to onset of activity in the next stroke 1a time of 50 % max mean voltage 2a time of max mean voltage 3a time of 50 % decline from max mean voltage Aa maximal mean voltage

Test muscle primary action (right digastric RD)

A *I* and thin mean voltage trace of *II* B full lines EMG 3 time to onset of activity EMG 4 time to cessation of activity 1b 2b 3b and Ab see 1a–3a and Aa in reference muscle

Time zero onset af activity in the reference muscle

BL (in A) base line of the mean voltage

RAT surface electrode RD needle electrode

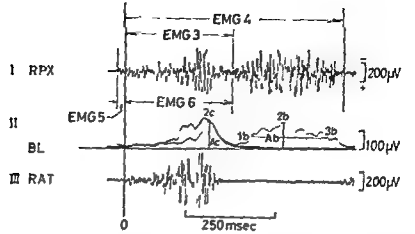


Fig 23b

To illustrate the evaluation of secondary activity in the test muscle during a chewing cycle (secondary activity activity appearing constantly in each chewing cycle in addition to the primary action which is the activity in the elevator muscles during closing and in the depressor and lip muscles during opening)

Test muscle (right external pterygoid RPX) *I* and thin mean voltage trace of *II*
Secondary activity EMG 5 time to onset of activity EMG 6 time to cessation of activity 2c time of max mean voltage Ac max mean voltage
Primary activity (EMG 3 and 4 1b 2b 3b and Ab) see Fig 23 a
Reference muscle (right anterior temporal RAT) *III* and fat mean voltage trace of *II*
Time zero onset of activity in the reference muscle BL base line of mean voltag
(RPX needle electrode RAT surface electrode)

activity in the right anterior temporal muscle Each chewing stroke was characterized by 12 time and amplitude parameters (Fig. 23 a) Secondary activity in the test muscle required 3–6 additional parameters (Fig 23 b)

In each subject the activity during a chewing stroke was characterized by average values of the time measurements and the maximal mean voltage from two (unilateral chewing) or three strokes (chewing of apple and bread) In general the first chewing strokes in each sequence of recording were se lected for measurement thus during natural chewing the first three strokes after biting off (1 2 and 3 Fig 24 a) The activity during the first and second stroke was sometimes irregular probably due to the positioning of the bolus and the measurements were then taken from the following strokes (3, 4 and 5 Fig 24 b) Recordings disturbed by movement artefacts (1 and 4 Fig 24 c) or with marked deviations of amplitude and time course (3 Fig 24 d) were excluded

The variation between the single strokes in series of 7–9 consecutive stro kes during natural chewing (given as the standard deviations in per cent of

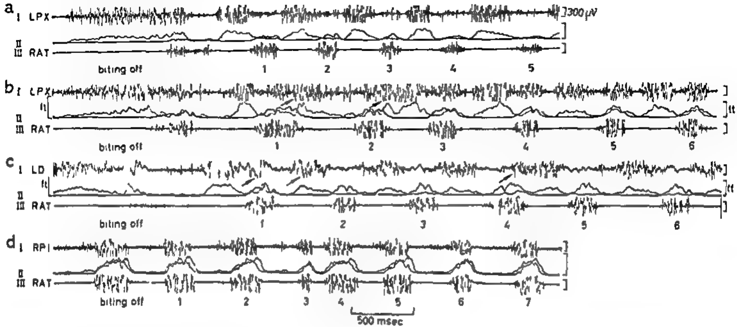


Fig 24

Selection of chewing strokes for measurement

a Example of a regular display of the activity in the test and reference muscle during natural chewing of apple the first three strokes *after biting off (1 2 and 3) were selected for measurement I and thin mean voltage trace of II left external pterygoid muscle* (LPX test m) *III* and fat mean voltage trace of *II* right anterior temporal muscle (RAT ref m) Subject 36 25 years old

b Example of irregular activity in the test muscle during the first two strokes after biting off (arrows) during natural chewing of apple the following three strokes (3 4 and 5) were selected for measurement *I* and thin mean voltage trace of *II* left external pterygoid muscle (LPX test m) *III* and fat mean voltage trace of *II* right anterior temporal muscle (RAT ref m) Subject 28 29½ year old

c Example of movements of the electrode in the test muscle during the first and fourth stroke (arrows) during natural chewing of apple the second third and fifth stroke were selected for measurement *I* and thin mean voltage trace of *II* left digastric muscle (LD test m) *III* and fat mean voltage trace of *II* right anterior temporal muscle (RAT ref m) Subject 28 29½ year old

d Example of marked deviation of the time course of the activity during the third stroke during natural chewing of bread the first second and fifth stroke were selected for measurement *I* and thin mean voltage trace of *II* right internal pterygoid muscle (RPI test m) *III* and fat mean voltage trace of *II* right anterior temporal muscle (RAT ref m) Subject 36 25 years old

Note difference between the calibration of the thin and fat trace in b and c (test muscles (I) needle electrodes reference muscle (III) surface electrodes)

Table 7

Variation in the time course and the maximal mean voltage during consecutive chewing strokes the activity in the right anterior temporal muscle during natural (A) and unilateral chewing (B)

	Subject no	No of strokes	Tot dur of activity msec		Time to 50% MVm x msec		Time to MVm x msec		Time to 50% decline from MVm x msec		MVm µV	
		n	M	SD	M	SD	M	SD	M	SD	M	SD
A Natural chewing (apple)	2	8	263	30 11%	101	60 60%	188	30 16%	238	35 15%	82	25 30%
	6	8	323	30 9%	90	55 60%	227	23 10%	315	25 8%	138	35 25%
	27	9	269	60 22%	37	24 65%	167	60 36%	257	22 9%	209	43 20%
B Unilateral chewing (right sided gum)	5	8	404	46 11%	173	30 17%	238	29 12%	305	42 14%	216	24 11%
	13	8	290	42 14%	143	20 14%	203	30 15%	266	55 21%	230	19 8%
	15	7	223	25 11%	97	25 26	157	30 19%	220	23 10%	150	11 7

M average SD standard deviation MV mean voltage Time zero onset of activity
The percentages indicate the standard deviations in per cent of the average values

the average values) was 10–30 per cent with respect to the total duration of activity the time of maximal activity the cessation of the period of strong activity and the maximal mean voltage (Table 7) The largest variation (60–65 per cent) was found for the time of onset of strong activity The variation between single strokes during unilateral chewing in most instances was 10–20 per cent i e less than during natural chewing The variation in the electrical activity from the first strokes after biting off to the last strokes before swallowing is discussed on p 97

A series of experiments comprised one set of data obtained on each test muscle and sixteen sets on the reference muscle

Swallowing The recordings during swallowing were evaluated as were the recordings during chewing except that each swallow was considered separately (Fig 25) Occasionally the activity tended to appear in two or three bursts but since it usually appeared continuously the total duration was described

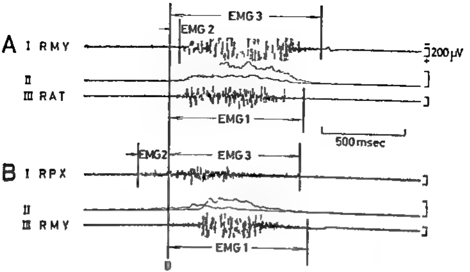


Fig 25

To illustrate the evaluation of the activity in the reference and test muscles during swallowing data obtained directly from the electromyograms (I and III) the mean voltage (II) was evaluated as during chewing (cf Fig 23 a)

Reference muscle (A right anterior temporal RAT B right mylohyoid RMY) EMG 1 total duration of activity

Test muscle (A right mylohyoid RMY B right external pterygoid RPX) EMG 2 time to onset of activity EMG 3 time to cessation of activity

Time zero onset of activity in the reference muscle (RAT surface electrode RMY and RPX needle electrodes)

The onset of activity during swallowing was often not as sharply delineated as during chewing Therefore to obtain uniform criteria for the measuring points all records from one subject were considered at the same time For some of the test muscles two sets of recordings were available one with the right anterior temporal and one with the right mylohyoid muscle as reference (see p 62) In each subject the calculation of the data from swallowing of saliva were based on two records and from swallowing of apple on one (selected as during chewing) each test muscle being represented by one set and the reference muscle by sixteen (right anterior temporal m) or seven (right mylohyoid m) sets of recordings

The measurements during chewing and swallowing were taken to the nearest millimetre With respect to time 1 mm corresponded to 5 msec in the case of the maximal mean voltage 1 mm equaled 4–20 μV depending on the gain of the mean voltage unit

Table 7

Variation in the time course and the maximal mean voltage during consecutive chewing strokes the activity in the right anterior temporal muscle during natural (A) and unilateral chewing (B)

	Subject no	No of strokes	Tot dur of activity msec		Time to 50% MV_{max} msec		Time to MV_{max} msec		Time to 50% decline from MV_{max} msec		MV_{max} µV	
		n	M	SD	M	SD	M	SD	M	SD	M	SD
A Natural chewing (apple)	2	8	263	30 11%	101	60 60%	188	30 16%	238	35 15%	82	25 30%
	[illegible]	8	323	30 9%	90	55 60%	227	23 10%	315	25 8%	138	35 25%
	27	9	269	60 22%	37	24 65%	167	60 36%	257	22 9%	209	43 20%
B Unilateral chewing (right sided gum)	5	8	404	46 11%	173	30 17%	238	29 12%	305	42 14%	216	24 11%
	13	8	290	42 14%	143	20 14%	203	30 15	266	55 21%	230	19 8%
	15	7	223	25 11%	97	25 26%	157	30 19%	220	23 10%	150	11 7%

M average SD standard deviation MV mean voltage Time zero onset of activity
The percentages indicate the standard deviations in per cent of the average values

the average values) was 10–30 per cent with respect to the total duration of activity the time of maximal activity, the cessation of the period of strong activity and the maximal mean voltage (Table 7) The largest variation (60–65 per cent) was found for the time of onset of strong activity The variation between single strokes during unilateral chewing in most instances was 10–20 per cent i e less than during natural chewing The variation in the electrical activity from the first strokes after biting off to the last strokes before swallowing is discussed on p 97

A series of experiments comprised one set of data obtained on each test muscle and sixteen sets on the reference muscle

Swallowing The recordings during swallowing were evaluated as were the recordings during chewing except that each swallow was considered separately (Fig 25) Occasionally the activity tended to appear in two or three bursts but since it usually appeared continuously the total duration was described

Table 8

Skewness and kurtosis of the distributions of time and amplitude data from the muscles of mastication obtained during chewing (A) *swallowing* (B) *at rest* (C) *and during full effort* (D)
Size of sample 36

		Number of distributions			
		A Chewing	B Swallowing	C Posture	D Full Effort
Skewness	$p\{\lvert\sqrt{b_1}\rvert\} > 0.05$	188	[illegible]	3	4
$\sqrt{b_1} = \frac{m_3}{m_2^{3/2}}$	$0.01 < p\{\lvert\sqrt{b_1}\rvert\} < 0.05$	24 (2)[1]	24 (2)	2	1
	$p\{\lvert\sqrt{b_1}\rvert\} < 0.01$	23 (3)	56 (6)	9	3
	Total	235	165	14	8
Kurtosis	$p\{a\} < 0.05$	208	129	7	7
$a = \frac{\text{mean deviation}}{\text{standard deviation}}$	$0.01 < p\{a\} < 0.05$	16 (1)[2]	22 (2)	2	0
	$p\{a\} > 0.01$	11	14 (1)	5	1
	Total	235	165	14	8

[1] Distributions with a negative skewness
[2] Leptokurtic distributions

tions the skewness was positive in 129 and negative in 13 distributions Among the 422 distributions 67 were platykurtic and only 4 leptokurtic Hence a positive skewness was the most frequent deviation from normality A classification of the deviations exceeding the one per cent limit of significance (Table 9) showed that more than half (26 of 43) of the distributions with values of $\lvert\sqrt{b_1}\rvert$ (skewness) larger than 1.50 originated from the recordings obtained during swallowing with respect to kurtosis (a) two thirds (22 of 31) of the non normal distributions had values of *a* close to the one per cent limit of significance

Differences between the electromyographic data obtained during natural function were tested by means of the t test The application of this test was considered justified since 1) the distribution of the arithmetic means of random samples has a skewness and kurtosis which is reduced proportionally to $1/\sqrt{n}$ as compared to the skewness and kurtosis of samples with *n* observations (*Croxton* 1959) and 2) that the comparisons in many instances involved distributions of the same shape i e distributions with the same degree of skewness and kurtosis Finally only few of the distributions with a marked

Table 9

Departure from normality (skewness and kurtosis) exceeding the 1 per cent level of significance distributions of time and amplitude data from the muscles of mastication obtained during chewing (A) *swallowing* (B) *at rest* (C) *and during full effort* (D) Size of sample 36

		Number of distributions			
		A Chewing	B Swallowing	C Posture	D Full Effort
Skewness $\sqrt{b_1}$	0.93–1.49	15 (1)[1]	30 (4)	2	1
$\sqrt{b_1} = \frac{m_3}{m}$	1.50–1.99	5 (2)	24 (2)	1	2
	2.00–2.49	3	1	4	0
	2.50–2.99	0	1	1	0
	3.00–3.49	0	0	1	0
	Total	23 (3)	56 (6)	9	3
Kurtosis [2]	0.71–0.68	8	9	3	1
$a = \frac{\text{mean deviation}}{\text{standard deviation}}$	0.67–0.64	2	2	1	0
	0.63–0.60	1	1	1	0
	0.59–0.56	0	1	0	0
	Total	11	13	5	1

[1] Distributions with a negative skewness.

[2] Platykurtic distributions there was only one leptokurtic distribution exceeding the 1 % level of significance (a = 0.89 1 % level 0.88)

departure from normality were used to establish differences in time and amplitude The distributions subjected to t-tests in spite of a skewness or kurtosis exceeding the one per cent limit of significance may be summarized as follows

1 Chewing The t-tests involved six skew distributions two of which were also platykurtic These distributions concerned the total duration of the chewing cycle (right ant. temporal m.) the onset of activity (internal pterygoid muscle) and the maximal mean voltage of the secondary activity in the external pterygoid muscles and in the muscles of the upper and lower lip Differences based on these distributions were highly significant ($p\ \{t\} < 0.005$) and can therefore hardly be invalidated by the deviations from normality

2 Swallowing Among 56 distributions with a marked skewness 22 were involved in t tests (4 platykurtic 1 leptokurtic) The non-normal distributions concerned mainly (a) the time of onset of activity and the onset of strong activity in the test muscles as referred to the onset of activity in the right anterior temporal muscle and (b) the maximal mean voltage in the masseter

and the external pterygoid muscles and the muscles of the upper and lower lip Conclusions with respect to these distributions were based on h ... significant differences ($p\{|t|\} < 0.005$)

3 Postural activity Differences between the activity with the mandible at rest and in the occlusal position were based on skew distributions in the case of the anterior ($p\{|t|\} < 0.001$) and posterior ($p\{|t|\} < 0.005$) temporal muscles

4 Full effort The mean voltage during maximal opening (external pterygoid and digastric m) and protrusion (digastric m) showed skew distributions However differences between the two positions were clearly demonstrated ($p\{|t|\} < 0.001$)

In the analysis of correlation between the electromyographic data and facial morphology logarithmic transformation was applied to normalize the positive skew distributions (see p 155)

Subjects

The 36 subjects*) were male dental students 20–30 years old (Fig 26) and had all teeth preserved (except the third molars), there was no history of orthodontic treatment and no signs or symptoms of neuromuscular disorder or involvement of the temporomandibular joints

The measurements describing the morphology of the facial skeleton and

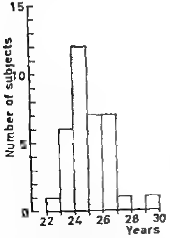


Fig 26
Age distribution of the 36 subjects examined

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*) Chosen at random from a group of 102 male dental students (Royal Dental College Copenhagen) selected by *Solow* (1966) for a morphological study of the facial skeleton

of the alveolar and dental arches presented by the 36 subjects appear in Tables 28, 29 and 30.

The experiments were carried out over a period of about two years and the time between the first and the last experiment on each subject is given in Table 10.

Table 10

Interval of time between the first and last experiment on each subject

Interval of time months	Number of subjects
>12	20
6–12	6
2–6	4
<2	6

MASTICATION

Elevator muscles

Anterior temporal muscles In the 36 subjects examined the total duration of the activity in *the right anterior temporal muscle* (reference muscle) varied from 180–380 msec the duration of the chewing cycle*) from 435–865 msec and the maximal mean voltage from 79–293 μV The activity in the reference muscle of the same subject on three different occasions is illustrated in Fig 27 Although similar in pattern the maximal mean voltage and the total duration of the activity and of the chewing cycle differed between subsequent strokes in each record as well as between the sessions In this subject the variation in the same session in per cent of the total variation (tot duration of activity 10 %, of chewing cycle 15 % and of the maximal mean voltage 16 %) was less than between sessions (tot duration of activity 25 % of chewing cycle 40 % and of the maximal mean voltage 25 %)

The variance analysis of the data obtained from the reference muscle was performed separately in each of the four types of chewing natural chewing of apple (a) or bread (b) unilateral chewing of gum on the right (c) or on the left (d) side The total duration of the electrical activity and of the chewing cycle as well as the maximal mean voltage and its time course differed significantly *($0.001 < p < 0.01$) from subject to subject* and from experiment to experiment on the same subject The variation between subjects was significantly larger than the individual variation ($p < 0.001$)

The variation in results obtained from the same subject at different sessions probably depended upon (1) the change in the position of the electrode and (2) whether or not simultaneous recording with intramuscular electrodes was used Duration and amplitude of the electrical activity tended to be smaller when only surface recording was used as compared to experiments in which recording was also intramuscular (cf Fig 27 where records A and B included simultaneous needle recording) However when analysing experiments with surface electrodes and with both surface and needle electrodes separately there was still a significant difference between experiments in the same subject

When considering data from the reference muscle obtained simultaneously with a pair of test muscles (data always obtained in the same session) the difference between single experiments was no longer significant Thus for an evaluation of the coordination of the single pair of test muscles and of the reference muscle during chewing the latter was characterized by the recordings obtained simultaneously with those of the pair of test muscles

*) The time difference between the onset of activity in the right ant temporal m in two consecutive chewing strokes

In unilateral chewing the activity in the right anterior temporal muscle was significantly larger during ipsilateral than during contralateral chewing (179 ± 8 µV and 139 ± 8 µV respectively) The 10 per cent prolongation of the chewing cycle in unilateral chewing as compared to natural chewing may be due to more extensive lateral movements or less automatic performance of one sided mastication

The time data obtained from the reference muscle were positively correlated in that the time to the onset the occurrence of the maximum and the cessation of the period of strong activity increased with the total duration of activity the maximal mean voltage was not correlated to the time pattern (Table 11)

The findings concerning the pattern of activity in the reference muscle (right anterior temporal muscle) were confirmed by the recordings from the *left anterior temporal muscle* (Fig 28 B, appendix Table II)

During *natural chewing* the two temporal muscles were on the average innervated symmetrically With respect to the left anterior temporal muscle the identical time patterns and the slightly larger maximal mean voltage when chewing bread (177 ± 9 µV) as compared to apple (160 ± 7 µV) are in keeping with the results from the reference muscle The coordination of the two anterior temporal muscles during natural chewing is exemplified in Fig 29 A in this subject the right muscle predominated slightly with respect to maximal mean voltage

The onset of the activity in the left muscle in relation to the right yielded information as to the uncertainty of the point used as a time reference In natural chewing the time difference between the onset of the activity in the two muscles was insignificant (appendix Table II) As the uncertainty in determining the onset of activity is equal for both muscles the standard error of the time difference divided by the square root of 2 (4 msec) indicates the error of the reference point

Unilateral chewing The variance analysis showed a *significant difference* between right and left sided chewing for all parameters Hence in unilateral chewing there was a time dispersal of the activity in the two temporal muscles the innervation of the ipsilateral preceding that of the contralateral by 10–20 msec The average maximal mean voltages showed a tendency to greater ipsilateral activity (ipsilateral 153 ± 8 µV contralateral 136 ± 9 µV) according to the variance analysis this predominance was systematic ($p < 0.01$) Two typical records from right and left sided chewing (Fig 29 B) show predominance of the muscle on the chewing side during the ascending phase of activity

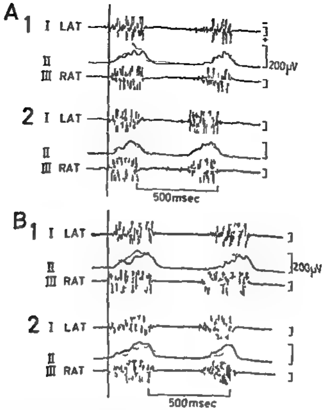


Fig 29

To illustrate the coordination of the right and left anterior temporal muscles during natural (A) *and unilateral* (B) *chewing*

I and fat mean voltage trace of *II* left anterior temporal muscle (LAT test m)

III and thin mean voltage trace of *II* right anterior temporal muscle (RAT ref m)

The vertical lines indicate the onset of activity in the reference muscle

A (nat chewing) note almost identical time patterns during chewing of apple (1) and bread (2) (Subject 15 26 years old)

B (right (1) and left (2) sided chewing of chewing gum) note predominance of the ipsilateral muscle during the ascending phase of activity (RAT in 1 LAT in 2 subject 24 24 years old)

Surface electrodes

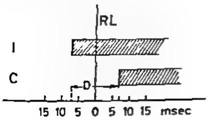
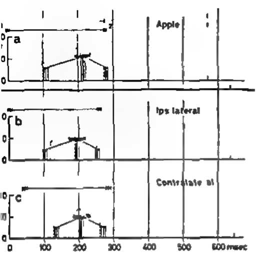


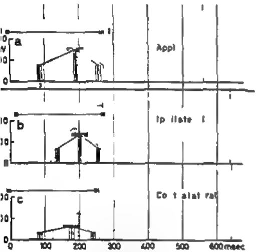
Fig 30

To illustrate the construction of the intermediate reference line (RL) *used as time zero of the activity in the test muscles during unilateral chewing* D represents the average time dispersal between the ipsilateral (I) and contralateral (C) onset of activity in the anterior temporal muscles during deliberately unilateral chewing

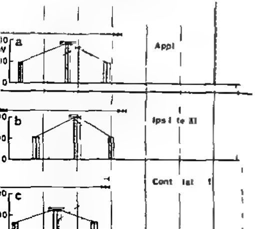
A Posterior temporal muscles



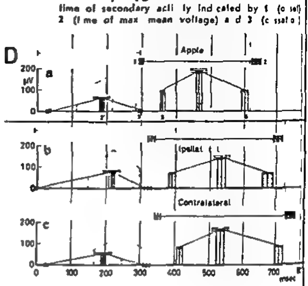
B Masseter muscles



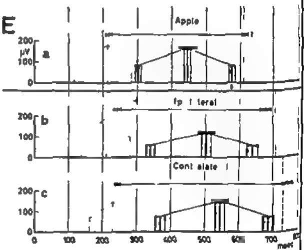
C Internal pterygoid muscles



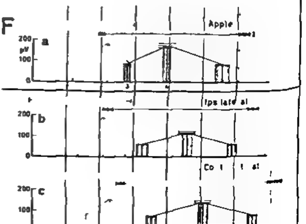
D External pterygoid muscles

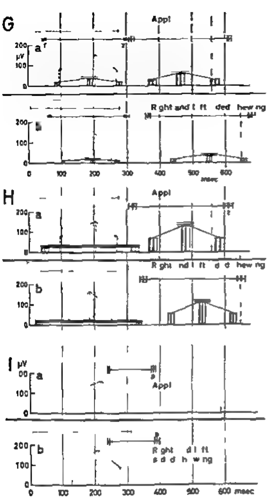


E Digastric muscles



F Mylohyoid muscles





G Muscles of the upper lip

s ond ry f v ty (1 2 3 4 d 5) d aled as the p m ry l ty (s e f g d) In b d t f om the ef e m cl e g v n both from ght (-----) a d left ( ) ded hew ng

H Muscles of the lower lip

o d ry l ty d t d by the o s t (1') d t o (2) d by th plat of th m olt eg l b d t f om th f e c m l g b th f om r ght (---) d left ( ) s ded h w ng

I Make (M) and break (B) of incisor contact

i b d t f om th f e m l g both f om ght (----) d l ft ( ) d d h w g

Fig 31

Average electrical activity in test muscles (A–H) and average time of make and break of incisor contact (I) during natural chewing of apple (a) and unilateral chewing of chewing gum (b and c) In elevator and depressor muscles (A–F) average data from right and left muscle of a pair (36 subjects)

The stipled lines indicate the average data from the right anterior temporal muscle (ref m) obtained simultaneously with those of the different test muscles (see p 75)

Time zero during chewing of apple onset of activity in the right anterior temporal muscle Time zero during unilateral chewing intermediate reference point halfway be tween the ipsilateral and contralateral onset of activity in the anterior temporal muscles (for definition see p 82 and Fig 30)

The uncertainty (average ±SE only indicated for test muscles) of the time data in indicated by the shaded areas of the maximal mean voltage by the largest distance be tween the horizontal lines on column *4*

1 onset of activity *2* cessation of activity *3* time of 50 % max mean voltage *4* time of max mean voltage *5* time of 50 % decline from max mean voltage The stipled line to the right indicates the cessation of the chewing cycle

The time dispersal between the onset of activity in the left and in the right muscle (appendix Table II) indicates that the reference point (the onset of activity in the right anterior temporal muscle) in the two series of experiments (right- and left sided chewing) occurred at different times in the chewing cycle For a joint treatment of the results obtained from the test muscles during right and left sided chewing, their activity was referred to a point half way between the onset of activity in the ipsilateral and contralateral anterior temporal muscles (Fig 30)

The results from the recordings of the *test muscles* are given as the average findings from each pair of symmetrical muscles and differences between the right and the left muscle of a pair are discussed separately

Posterior temporal muscles (Fig 31 A appendix Table III) The activity in the posterior part of the temporal muscle during *natural chewing* occurred almost simultaneously with that from the anterior part, the only difference being the significantly smaller mean voltage of the former (apple ant part 156 ± 6 μV post part 110 ± 5 μV bread ant part 184 ± 6 μV, post part 141 ± 6 μV) This difference is probably due to the smaller volume of the posterior part The coordination of the two parts of the temporal muscle during natural chewing is exemplified in one subject (Fig 32 A) in whom the mean voltage in the anterior part (thin trace) was greater than in the posterior (fat trace) during the ascending phase of activity The two muscles were not entirely relaxed between the chewing strokes the intermediary activity being most pronounced in the posterior part

In *unilateral chewing* the time dispersal of the activity was more pronounced in the posterior than in the anterior part thus at the onset and in the ascending phase the ipsilateral action was significantly (30–40 msec) ahead of the contralateral On the average the maximal mean voltage was the same in both series (ipsilateral 94 ± 5 μV contralateral 99 ± 5 μV) in contrast to the findings in the reference muscle Thus in unilateral chewing the identical maximal mean voltages in the ipsilateral and contralateral muscle (cf Fig 32 B) and the difference in time indicated an innervation of the horizontal fibres of the temporal muscle independent of that of the vertically directed fibres

Masseter muscles (Fig 31 B appendix Table IV) During *natural chewing* the masseter muscles were innervated simultaneously with the right anterior temporal muscle (Fig 33 A) and as in this muscle chewing of bread was associated with the largest maximal mean voltage (bread 173 ± 7 μV apple 143 ± 6 μV)

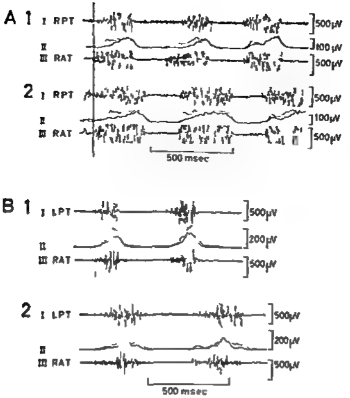


Fig 32

To illustrate the coordination of the posterior and anterior temporal muscles during natural (A) *and unilateral chewing* (B)

A *I* and fat mean voltage trace of *II* right posterior temporal muscle (RPT test m) *III* and thin mean voltage trace of *II* right anterior temporal muscle (RAT ref m) Note the predominance of the reference muscle in the ascending phase of activity during chewing of apple (1) and bread (2) (subject 24 25 years old)

B *I* and thin mean voltage trace of *II* left posterior temporal muscle (LPT test m) *III* and fat mean voltage trace of *II* right anterior temporal muscle (RAT ref m) Note that the maximal mean voltage in the test muscle was about the same during right (1) and left (2) sided chewing while that in the reference muscle was greatest in *I* (subject 22 25 years old)

The vertical lines indicate the onset of activity in the reference muscle (surface electrodes)

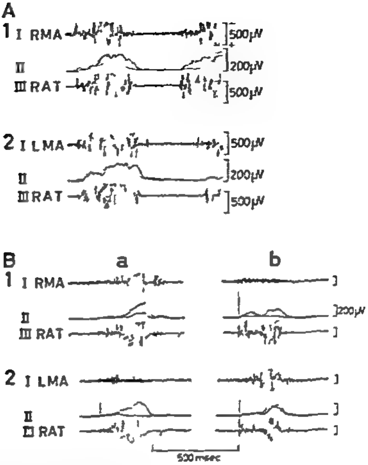


Fig 33

To illustrate the coordination of the masseter muscles and the reference muscle during natural (A) *and unilateral* (B) *chewing*

I and thin mean voltage trace of *II* right (1 RMA) and left (2 LMA) masseter muscles (test m.)

III and fat mean voltage trace of *II* right anterior temporal muscle (ref m.)

A note the simultaneous innervation of test and reference muscles (subject 27 26 years old)

B note the difference between the time course and degree of activity in the ipsilateral and contralateral masseter during right (a) and left (b) sided chewing (subject 23 25½ year old)

The vertical lines indicate the onset of activity in the reference muscle (surface electrodes)

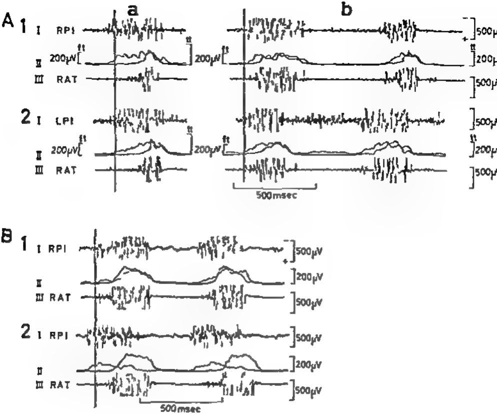


Fig. 34

To illustrate the coordination of the internal pterygoid muscles and the reference muscle during natural (A) *and unilateral* (B) *chewing*

I and thin mean voltage trace of *II* right (A1 and B RPI) and left (A2 LPI) internal *pterygoid muscles (test m.)*

III and fat mean voltage trace of *II* right anterior temporal muscle (RAT ref. m.)

A note the predominance of the test muscle in the ascending phase of activity during chewing of apple (a) and bread (b) (difference in calibration of fat (ft) and thin (tt) trace subject 33 25 years old)

B note the difference between the time course and the degree of activity in the test muscle during right (1 ipsilat.) and left (2 contralat.) sided chewing (subject 29 24½ year old)

The vertical lines indicate the onset of activity in the reference muscle (RAT surface electrodes RPI and LPI needle electrodes)

During *unilateral chewing* the ipsilateral and the contralateral action differed with respect to duration timing and maximal mean voltage Thus the contralateral activity in the masseter muscles appeared 30 msec before the ipsilateral ($p < 0\ 005$), had a longer duration of the period of strong activity and a smaller maximal mean voltage ($p < 0\ 001$ ipsilateral period of strong activity 120 msec maximal mean voltage 137 μV, contralateral 160 msec and 63 μV respectively) The substantial difference between the degree of activity on the chewing side as compared to the balancing side is exemplified in Fig 33 B

Internal pterygoid muscles (Fig 31 C, appendix Table V) In all types of chewing the internal pterygoid muscles were the first elevators to be innervated during the closing movement The onset of their activity was 30–40 msec ahead of the reference muscle ($p < 0\ 001$) except during ipsilateral chewing The total duration and the interval of strong activity lasted for about 100 msec longer than in the reference muscle The early onset of strong activity in the internal pterygoid muscles during natural chewing as compared to that of the reference muscle is exemplified in Fig 34 A The right and left internal pterygoid muscles in this subject differed with respect to intermediary activity During *natural chewing* the maximal mean voltages from apple (189 ± 5 μV) and bread (200 ± 5 μV) were identical During *unilateral chewing* these muscles revealed a distinct difference between ipsi and contralateral activity (Fig 34 B) with respect to timing (contralateral lead of period of strong activity 30–60 msec $p < 0\ 001$) and to maximal mean voltage (ipsilateral 203 ± 7 μV contralateral 126 ± 8 μV)

Depressor muscles

External pterygoid muscles The external pterygoid muscles were activated twice in each chewing cycle the primary action occurred in the opening movement and was always distinct the secondary was coordinated with the activity in the elevator muscles and was variable in intensity (Fig 35 A and B)

The average results (Fig 31 D appendix Table VI) showed for all types of chewing a prolongation of the chewing cycle with this test muscle as compared to the average (6–8 per cent) probably because of a retarding effect of the intraorally inserted needle electrode The primary (opening) activity was larger than the secondary in all types of chewing

As regards the primary action during *natural chewing* the bread caused prolongation of the descending phase of about 60 msec while the maximal mean voltage (199 ± 5 μV) was about the same as during chewing of apple (186 ± 5 μV) In the closing movement (secondary action) the maximal ac

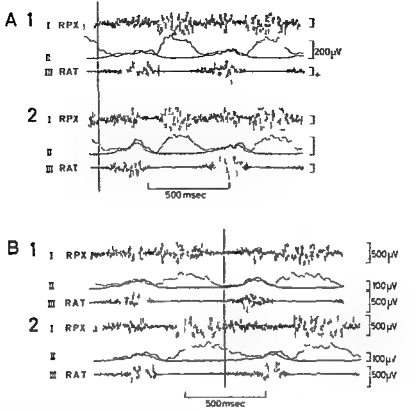


Fig 35

To illustrate the coordination of the external pterygoid muscle and the reference muscle during natural* (A) *and unilateral* (B) *chewing

I and thin mean voltage trace of *II* right external pterygoid muscle (RPX test m) *III* and fat mean voltage trace of *II* right anterior temporal muscle (RAT ref m)

A note that the primary activity in the test muscle (between actions of the reference m) was about the same during chewing of apple (1) and bread (2) while the secondary activity (simultaneously with the reference m) was strongest when bread was being chewed

B note that the primary activity was slightly lower during right sided (1 ipsilateral) than during left sided (2, contralateral) chewing

The vertical lines indicate the onset of activity in the reference muscle (RAT surface electrodes RPX needle electrodes subject 36 25 years old)

tivity occurred 30 msec before the maximum in the reference muscle in both types of chewing and the maximal mean voltage was larger during chewing of bread (89 ± 4 µV) than during chewing of apple (64 ± 4 µV). The electromyograms of Fig. 35 A show about the same activity during opening in both types of chewing and less secondary activity when chewing apple (1) as compared to bread (2).

Unilateral chewing was characterized by a simultaneous occurrence of maximal activity in the two sides during opening, the degree of activity being largest in the contralateral muscle (contralateral 167 ± 5 µV, ipsilateral 145 ± 5 µV). The secondary activity was greatest in the ipsilateral muscle, 79 ± 4 µV as compared to 55 ± 3 µV in the contralateral.

Digastric muscles. The primary activity in the digastric muscles occurred in the opening phase and the onset of their action occurred near the maximum of activity in the reference muscle; there was a continuous discharge between the primary actions, more pronounced during natural chewing of apple and bread than during unilateral chewing (Fig. 36).

The average results (Fig. 31 E, appendix Table VII) showed that the digastric preceded the primary action of the external pterygoid muscles at the onset by 80–100 msec ($p < 0.001$) and at the onset of strong activity by 40–60 msec ($p < 0.01$). The close association between the onset of the activity in the digastric muscles and the time of maximal innervation of the temporal muscle appeared in the individual subjects as well; the time to onset of activity in the digastric muscles increased with the time to maximal activity in the reference muscle (coefficient of correlation $r = + 0.40$, $0.01 < p < 0.05$).

During *natural chewing* the digastric muscles were innervated with the same intensity irrespective of the food item, but in accordance with the extended opening movement the total duration of the activity was largest when chewing bread (apple 395 msec, bread 445 msec). During *unilateral chewing* there was a slight time dispersal, the strongest part of the ipsilateral action occurring 30–50 msec before the contralateral ($p < 0.05$). Furthermore the maximal mean voltage was greatest in the contralateral muscle (ipsilateral 120 ± 5 µV, contralateral 149 ± 6 µV).

Mylohyoid muscles. There was a striking resemblance between the average results from the mylohyoid (Fig. 31 F, appendix Table VIII) and the digastric (Fig. 31 E) muscles, but maximal activity occurred first in the mylohyoid muscles (time dispersal 40–50 msec, $p < 0.05$).

During *natural chewing* the activity in the mylohyoid muscles preceded that of the digastric muscles, the interval of strong activity in the mylohyoid

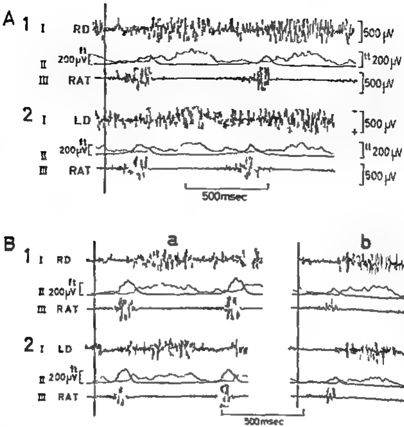


Fig 36

To illustrate the coordination of the digastric muscles and the reference muscle during natural (A) *and unilateral* (B) *chewing*

I and thin mean voltage trace of *II* right (1 RD) and left (2 LD) digastric muscles test m)

III and fat mean voltage trace of *II* right anterior temporal muscle (RAT ref m)

Note that the intermediary activity in the test muscles was larger during natural chewing of apple (A) than during right (B a) and left (B b) sided chewing of chewing gum note also that the onset of activity in the digastric muscles coincided with maximal activity in the reference muscle (difference between calibration of fat (ft) and thin (tt) trace subject 33 25 years old)

The vertical lines indicate the onset of activity in the reference muscle (RAT surface electrodes RD and LD needle electrodes)

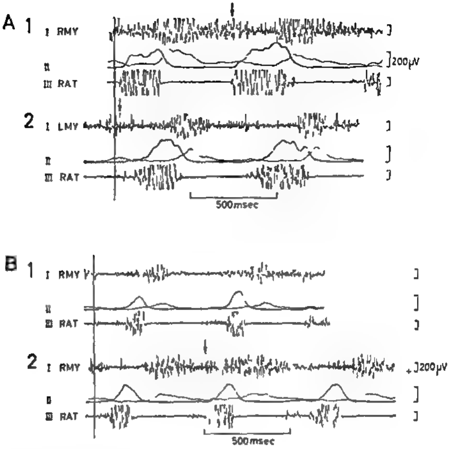


Fig. 37

To illustrate the coordination of the mylohyoid muscles and the reference muscle during natural (A) *and unilateral* (B) *chewing*

I and thin mean voltage trace of *II*: right (A1 and B: RMY) and left (A2: LMY) mylohyoid muscle (test m.)

III and fat mean voltage trace of *II*: right anterior temporal muscle (RAT: ref. m.)

Note that the onset of strong activity in the test muscle coincided with the last part of the strong action of the reference muscle during chewing of apple (A) while the time dispersal between the activity in the two muscles was larger during right (B1) and left (B2) sided chewing. Note also that the intermediary activity sometimes appeared in separate bursts (arrows in A1 and 2) and sometimes as a continuous discharge between the primary actions (arrow in B2).

The vertical lines indicate the onset of activity in the reference muscle (RAT: surface electrodes, RMY and LMY: needle electrodes, subject 32, 24 years old).

muscles coincided with the last part of the strong activity in the reference muscle (Fig 37 A) During *unilateral chewing* (Fig 37 B) the contralateral activity exceeded the ipsilateral (contralateral 141 ± 6 μV ipsilateral 117 ± 6 μV)

In addition to the primary (opening) activity, the mylohyoid muscle showed intermediary activity This was irregular and unsuitable for a quantitative evaluation sometimes it appeared as a separate burst of activity (Fig 37 A) at other times as a constant discharge between the primary actions (Fig 37 B)

In comparison with the other depressor muscles the correlation between the maximal mean voltages in the right and the left mylohyoid muscle was most pronounced probably because they anatomically are more intimately connected (coefficients of correlation between the right and left muscle ext pterygoid m $r = +0\,35$ digastric m $r = +0\,40$, $0\,01 < p < 0\,05$, mylohyoid m $r = +0\,70$ $p < 0\,001$)

Orbicularis oris muscles

The activity in the orbicularis oris muscle was recorded from the upper and lower lip close to the midline on the right side The primary action occurred in the opening movement overlapping somewhat with the onset of the activity in the reference muscle in the next stroke (Fig 38) With respect to the time course the pattern of activity in the upper lip was difficult to evaluate uniformly However since some increase in activity was present both during opening and closing the usual parameters of the mean voltage were used as a rough estimate of the time course during both movements The electromyograms from the lower lip were more suitable for a uniform quantitative evaluation They showed distinct bursts of activity during opening (primary activity) and a rather constant discharge during closing (secondary activity)

In the variance analysis the results from the upper and lower lip were treated as the rest of the test muscles i e in a three way classification (see p 70) On account of significant differences the patterns of the two muscles were considered separately (upper lip Fig 31 G appendix Table IX, lower lip Fig 31 H appendix Table X) In each muscle the activity during right and left sided chewing was about the same and therefore the results from these experiments were combined

During *natural chewing* the primary activity in the upper and lower lip differed with respect to the intensity of the discharge the maximal mean voltage in the lower lip (139 ± 8 μV) being twice that in the upper (64 ± 4 μV) the degree of activity in the closing movement was almost the same i e 30–40 μV In *unilateral chewing* the onset of activity in the lips was

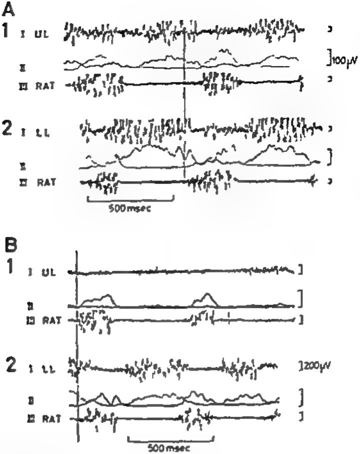


Fig 38

To illustrate the coordination of the muscles of the lips and the reference muscle in a subject with great* (A) *and a subject with slight* (B) *activity in the upper lip during natural chewing of apple

I and thin mean voltage trace of *II* upper (1 UL) and lower (2 LL) lip muscles (test m)

III and fat mean voltage trace of *II* right anterior temporal muscle (RAT ref m)

A. note the strong (primary) activity in both lips between the actions of the reference muscle and the distinct (secondary) activity in the upper lip simultaneously with this muscle (subject 11 24¹ year old)

B note the slight activity in the upper lip between (primary) and especially simultaneously with (secondary) the action of the reference muscle (subject 13 25 years old)

The vertical lines indicate the onset of activity in the reference muscle (surface electrodes)

delayed about 100 msec consistent with the extended opening phase and the intensity was reduced by 40 per cent in the upper and 15 per cent in the lower lip

That the electromyograms obtained from the lips were compound recordings from various separately innervated muscles was reflected in a large variation in the time relation of their activity Thus the upper lip was just as frequently innervated first as the lower Only in 2 of 36 subjects did the activity in the upper lip slightly exceed that in the lower during chewing of bread

Variation in the individual subject

Reference muscle The first column in Table 12 shows the standard deviations of the difference between two sets of measurements performed on the same recordings from the reference muscle (12 subjects) The measurements comprised three complete chewing cycles in each subject and the differences were based on the average values obtained in the three cycles

The data of time and amplitude of the activity in the reference muscle in two experiments in the same session (Table 12 column 2) differed 4–7 times more than to be expected if they were caused solely by errors of measuring, hence the masticatory activity itself varied

Table 12

Standard deviations of mean differences between time and amplitude data of the reference muscle (a) *obtained from two sets of measurements on the same record and* (b) *between two recordings obtained in the same and* (c) *in two subsequent sessions (natural chewing of apple)*

Parameters	Standard deviations of mean differences		
	a Same record measured twice	b Same session	c Different sessions
Tot dur of activity msec	10	41	53
Time to [illegible] % MV_m msec	7	39	61
Time to MV_m msec	5	39	64
Time to 50 % decline from MV_m msec	8	38	50
MV_m µV	4	31	51

MV mean voltage a 12 subjects b and c 36 subjects
Time zero onset of activity

The third column was based on differences between an experiment carried out with surface electrodes alone and one involving a change in the position of the electrode and simultaneous needle recording. This caused a 30–50 per cent increase ($0.01 < p < 0.05$) in the variation of the pattern of the reference muscle.

Coordination of the right and left muscle of a symmetrical pair. The variation in the coordination of the right and left anterior temporal muscle during natural chewing of apple in a single subject was determined in three ways:

1. Directly, on a simultaneous recording from the two muscles. The difference between corresponding parameters was measured directly, i.e. not involving the reference point.
2. Indirectly, on a simultaneous recording with the onset of activity in the right muscle as reference point, i.e. the measuring procedure otherwise applied.
3. Indirectly, using the onset of activity in the right anterior temporal muscle as reference, but in the case of the right muscle using data from another experiment in the same session. This procedure was performed to have a direct comparison of the conditions prevailing when calculating differences between the right and the left of the other muscles.

With respect to total duration of activity and the time to the onset and cessation of strong activity, the difference between the right and left anterior temporal muscles varied twice as much when obtained from two different recordings (3) than when determined from a simultaneous recording (1 and 2).

Table 13

Standard deviations of mean differences between (a) *the maximal mean voltage and* (b) *the time of its occurrence in the right and left muscle of a symmetrical pair* (natural chewing of apple, 36 subjects)

Pairs of muscles		Ant. temp.	Post. temp.	Masseter	Int. pterygoid	Ext. pterygoid	Digastric	Mylohyoid
Standard deviations of mean differences	a. Max. mean voltage µV	40	39	68	43	51	65	65
	b. Time to max. mean voltage msec	44	60	51	99	68	91	113

Time zero in *b*: onset of activity in the right ant. temporal muscle.

The difference between the right and left muscle with respect to the time of maximal activity and the maximal mean voltage showed the same variation (given for elevator and depressor muscles in Table 13) irrespective of the method of evaluation The largest time dispersal of maximal activity in the elevator muscles occurred in the internal pterygoid muscles (SD 99 msec) the mylohyoid muscles showed the largest difference in the depressor group (SD 113 msec) As regards the maximal mean voltage the two masseter muscles differed 60–70 per cent more than the temporal and the internal pterygoid muscles in spite of the intramuscular recording from the internal pterygoid muscles

The ipsilateral and contralateral patterns of most muscles differed significantly with respect to the degree of activity A larger maximal mean voltage in the right or the left muscle with both food items during natural chewing could indicate either the presence of a preferred side of chewing or be due to the position of the electrode In the case of a randomly changing side of chewing a persisting predominance would be expected in half the subjects A chi square test was applied to evaluate the significance of the observed number of subjects with persisting unilateral predominance (Table 14) Only in the case of the external pterygoid muscles (primary activity) did this test show a significant predominance of one muscle in both types of natural chewing In the elevator muscles a difference between the right and

Table 14

Number of subjects with persisting and with changing predominance of the right or left muscle of a symmetrical pair during natural chewing of apple and bread (total 36 subjects)

	Muscles				
	Anterior temporal	Masseter	Int pterygoid	Ext pterygoid[2]	Digastric and Mylohyoid
Persisting predominance number of subjects	23	19	22	29	20
Changing predominance number of subjects	12	14	14	7	15
χ [1]	3 46 $p>0\,05$	0 76 $p>0\,5$	1 78 $p>0\,1$	13 46 $p<0\,001$	0 72 $p>0\,6$

[1] Chi square values calculated with the assumption of an equal number of subjects with persisting and changing side of predominance

[2] Primary action

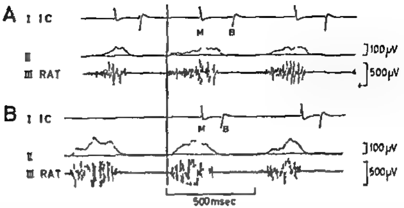


Fig 40

To illustrate the relation in time between incisor contact (*I* IC) *and the electrical activity in the right anterior temporal muscle* (RAT *II* mean voltage *III* electromyogram) *during natural chewing of apple* (A) *and bread* (B) *I* make (M) and break (B) of contact The vertical line indicates the onset of activity in the reference muscle (subject 2 23½ year old surface electrodes)

Simultaneous recordings of the activity in the reference muscle and of tooth contact in the incisor region were obtained in all 36 subjects (Fig 40) In addition it was possible to record molar contact on one side in seven and on both sides in three subjects thus thirteen experiments were available for the evaluation of the timing of the molar contacts

Tooth contact during mastication was observed in nearly all experiments When recording in the incisor region contact was absent in one of the 36 subjects when chewing was performed on the left side, when recording from the side regions contact was absent on the balancing side in one subject Contact between the upper and lower incisors during biting off occurred in two thirds of the subjects when chewing apple in 50 per cent when chewing bread The molar contacts showed the same distribution (Table 15)

Table 15

Incidence of tooth contact during biting off

Region of contact	Number of recordings with contact *Apple*	*Bread*	*N*
Incisors	23	17	36
Molars	10	5	13

N = 36 recordings in 36 subjects
N = 13 indicates 13 recordings in 10 subjects

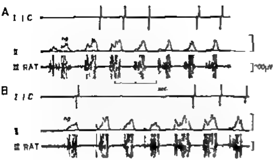


Fig 41

To illustrate that tooth contact occurred at an earlier stroke during chewing of apple (A) *than during chewing of bread* (B)

Simultaneous recordings of incisor contact (*I* IC) and the electrical activity in the right anterior temporal muscle (RAT *II* mean voltage *III* electromyogram) during the first 6 chewing strokes (1–6) after biting off (subject 23 25½ year old surface electrodes)

Table 16

Incidence of tooth contact during chewing

Region of contact	Stroke no	Number of subjects with contact				N
		Apple	Bread	right sided	left sided	
Incisor	1	25	9	36	34	36
	2	24	16	34	35	36
	3	29	14	36	35	36
	4	28	19	34	34	36
	5	30	19	–	–	36
Molar	1	8	7	12	13	13
	2	9	8	11	13	13
	3	10	8	12	12	13
	4	10	6	12	13	13
	5	10	6	–	–	13

N = 36 recordings in 36 subjects
N = 13 indicates 13 recordings on 10 subjects

Tooth contact occurred at an earlier stroke when chewing apple than when chewing bread (Fig 41) As regards natural chewing the incidence of tooth contact between upper and lower incisors increased in the course of the chewing of both types of food (apple from 70 to 80 per cent bread from 25 to 50 per cent) in unilateral chewing contact was present in practically all strokes (Table 16) The findings from recording contact in the molar regions were consistent with those in the incisor area

Tested with respect to differences between subjects and types of chewing the time of make and break of incisor contact showed a significant difference only between subjects ($p < 0.001$) The average results (Fig 31 I) demonstrated that contact between the upper and the lower teeth lasted 130–140 *msec in each chewing cycle i e during 20 per cent of its total duration*

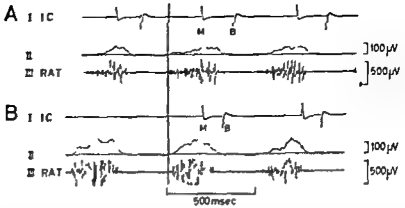


Fig 40

To illustrate the relation in time between incisor contact (I IC) and the electrical activity in the right anterior temporal muscle (RAT *II* mean voltage *III* electromyogram) *during natural chewing of apple* (A) *and bread* (B) *I* make (M) and break (B) of contact The vertical line indicates the onset of activity in the reference muscle (subject 2 23½ year old surface electrodes)

Simultaneous recordings of the activity in the reference muscle and of tooth contact in the incisor region were obtained in all 36 subjects (Fig 40) In addition it was possible to record molar contact on one side in seven and on both sides in three subjects thus thirteen experiments were available for the evaluation of the timing of the molar contacts

Tooth contact during mastication was observed in nearly all experiments When recording in the incisor region contact was absent in one of the 36 subjects when chewing was performed on the left side when recording from the side regions contact was absent on the balancing side in one subject. Contact between the upper and lower incisors during biting off occurred in two thirds of the subjects when chewing apple in 50 per cent when chewing bread The molar contacts showed the same distribution (Table 15)

Table 15

Incidence of tooth contact during biting off

Region of contact	Number of recordings with contact Apple	Bread	N
Incisors	23	17	36
Molars	10	5	13

N = 36 recordings in 36 subjects
N = 13 indicates 13 recordings in 10 subjects

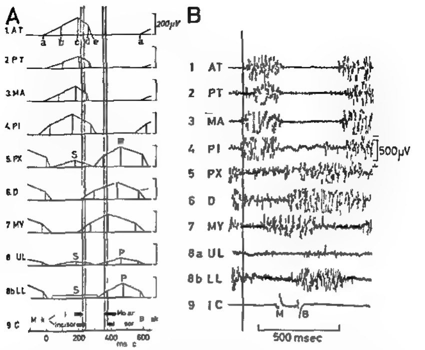


Fig 42

To illustrate the relation in time between the electrical activity in the elevator (1–4) *depressor* (5–7) *and lip* (8) *muscles and the occurrence of tooth contact* (9) *during natural chewing*

A Average of electrical activity (36 subjects) and of make and break of incisor (36 subjects) and molar (10 subjects) contact *a* onset of activity *b* time of 50 % max mean voltage *c* time of max mean voltage *d* time of 50 % decline from max mean voltage *e* cessation of activity In 5 and in 8a and 8b *S* indicates the secondary *P* the primary action Time zero onset of activity in the right anterior temporal muscle

B Recordings of the electrical activity and of the make (M) and break (B) of incisor contact from one subject (no 13 25¹/ year old) The vertical line indicates the onset of activity in the right anterior temporal muscle (1–3 and 8a and 8b surface electrodes 4–7 needle electrodes)

AT ant temp PT post temp MA masseter PI int pterygoid PX ext pterygoid MY mylohyoid UL upper lip LL lower lip C tooth contact in *B* IC incisor contact

temporal and masseter muscles the activity in the elevators began to decrease shortly before molar contact was made and reached the resting level in the first part of the interval of contact Of the *depressor muscles* the mylohyoid muscles were activated first immediately followed by the digastric muscles and the make of tooth contact Activation of the external pterygoid muscles (primary action) did not occur until the middle of the interval of contact was reached i e at the end of activity in the elevator muscles The time dispersal between the onset of activity in the depressor muscles indicated that opening was asso-

Table 18

Coefficients of correlation (r) between time and amplitude data of the right anterior temporal muscle and the time of make and break of incisor contact during natural chewing of apple (36 subjects)

Tooth contact (incisor region)	Electrical activity in the right ant temporal muscle					
	Tot dur of activity msec	Tot dur chewing cycle msec	Time to 50 / MV_{max} msec	Time to MV_{max} msec	Time to 50 / decline from MV_{max} msec	MV_{max} µV
Time to make of contact msec	+0 24	+0 06	+0 00	+0 17	+0 20	−0 13
Time to break of contact msec	+0 62***	+0 46**	+0 41*	+0 58***	+0 60 **	+0 14

r* 0 01<p<0 05 r** 0 001<p<0 01 r*** p<0 001
MV mean voltage Time zero onset of activity in the right ant temporal muscle

ciated in the beginning with a lowering of the mandible translation being confined to a later phase The period of strong activity in the *muscles of the lips* (primary action) occurred simultaneously with that in the depressors This timing of lip action serves to seal the oral cavity *Tooth contact* occurred in three stages molar contact then contact in all areas and finally it was confined to the incisor region

Neither the time course nor the maximal mean voltage in the reference muscle showed a systematic relation with the onset of tooth contact (Table 18) The time course but not the maximal mean voltage was positively correlated to the time of break of tooth contact Hence duration of the interval of contact depended on the duration of the activity but not on its intensity

Relation in time between the activity in the elevator and depressor muscles and tooth contact during *unilateral chewing* is shown in Fig 43 A (average findings) and B (recordings from one subject) In the *elevator* muscles the activity occurred first in the ipsilateral temporal muscles while activity led in the contralateral masseter and internal pterygoid muscles In addition there was an ipsilateral predominance in the masseter and internal pterygoid muscles The electromyograms (B) showed the time dispersal in the case of the posterior temporal (2) and masseter (3) muscles, the anterior parts of the temporal muscle (1) were innervated symmetrically in the internal pterygoid muscles (4) the ipsilateral action was maintained for a longer period of time than the contralateral The maximal mean voltage

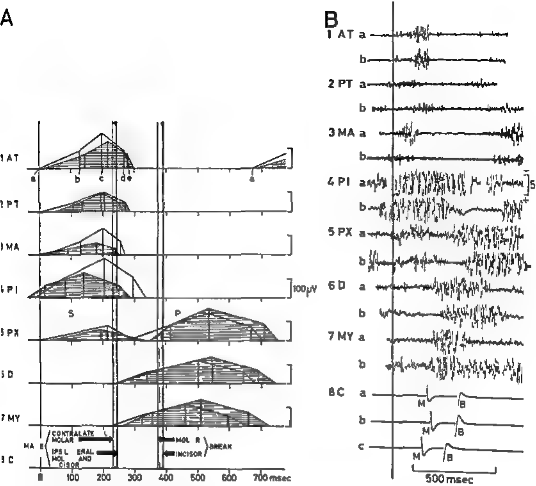


Fig 43

To illustrate the relation in time between the electrical activity in the elevator (1–4) *and depressor* (5–7) *muscles and the occurrence of tooth contact* (8) *during unilateral chewing of chewing gum*

A Average of electrical activity (36 subjects) and of make and break of incisor (36 subjects) and molar (10 subjects) contact shaded areas indicate contralateral activity *a* onset of activity *b* time of 50 % max mean voltage *c* time of max mean voltage *d* time of 50 % decline from max mean voltage *e* cessation of activity In 5 *S* indicates the secondary *P* the primary action Time zero intermediate reference point half way between the ipsilateral and contralateral onset of activity in the anterior temporal muscles (1 AT for definitions see p 82 and Fig 30)

B Recordings of the electrical activity (1–7 *a* ipsilateral *b* contralateral) and of the make (M) and break (B) of tooth contact (8C *a* incisor contact bands *b* ipsilateral molar *c* contralateral molar) from one subject (no 18 27½ year old) during right sided chewing of chewing gum The vertical line indicates the onset of activity in the right (ipsilateral) anterior temporal muscle (1–3 surface electrodes 4–7 needle electrodes)

AT ant temp PT post temp MA masseter PI int pterygoid PX ext pterygoid D digastric MY mylohyoid C tooth contact
Note time scale in A is twice that in B

in the contralateral *depressor muscles* was 15–20 per cent larger than the ipsilateral but the ipsilateral action occurred earlier (the electromyograms in B do not show the slight difference in degree of activity) The depressor muscles were activated in the same sequence as during natural chewing at first the mylohyoid, then the digastric and finally the external pterygoid muscles (primary activity) Contact on the contralateral side preceded that on the ipsilateral side (and on the incisors) in Fig 43 B by about 30 msec

Discussion

Muscle activity

The findings as regards the activity in *the anterior temporal muscles* during unilateral chewing are in keeping with previous observations (*Perry* and *Harris* 1954 *Perry* 1955 and 1961 *Ahlgren* 1966) repeated recordings with different positions of the electrodes showed a predominance of the muscle on the chewing side (appendix Table I), in addition this muscle was activated first (appendix Table II) In my group of adults the total duration of the chewing cycle (difference in time between the onset of activity in the anterior temporal muscle in two subsequent strokes 680 msec) was slightly shorter than in the children investigated by *Ahlgren* (1966 750 msec), the duration of the activity in the temporal muscle was the same in both groups (300 msec)

Recordings in the *posterior part of the temporal muscle* during free lateral movements of the mandible have shown that it is activated on the side towards which the movement takes place (*Carlsoo* 1952 *Gopfert* and *Gopfert* 1955 *Zenker* and *Zenker* 1955 *Greenfield* and *Wyke* 1956 and *Woelfel* et al 1960) With this observation in mind my findings during unilateral chewing indicate that the posterior part of the ipsilateral temporal muscle is engaged in stabilizing the mandible toward the chewing side and the delayed contralateral action directs the mandible toward the intercuspal position in the final part of the chewing stroke

My average findings are in keeping with those of *Ahlgren* (1966) that the right and left temporal muscles during natural chewing display the same degree of activity and attain maximal activity at the same time However, differences between the right and left muscle in the single subject were considerable (Table 13)

Attempts have been made to distinguish electromyographically the function of the superficial and deep portion of the *masseter muscle* With intramuscular recording *Carlsoo* (1952) showed that the deep portion contrary to the superficial was active during translation from the contralateral side

to rest position The larger superficial portion covers (see p 17) the vertically directed profound fibres except for a small area in front of the temporomandibular joint Therefore if recording is by means of surface electrodes (*Greenfield* and *Wyke* 1956 *Eschler* 1958 *Krazer* 1960 *Witt* 1961 1963) a differentiation of the function of the two parts seems hardly possible

In my experiments no attempt was made to differentiate between the activity in the superficial and deep portions of the muscle During unilateral chewing the contralateral muscle was activated earlier but less than the ipsilateral This is consistent with previous observations (*Perry* and *Harris* 1954 *Perry* 1955 and 1961 *Pruzansky* 1958 *Ahlgren* 1966)

A preferred side of action during natural chewing often observed in children (*Eschler* 1955) did not appear in my group of adults In most subjects the predominant side changed at random from one sequence of chewing strokes to the other and (in keeping with *Ahlgren* 1966) the average degree of activity in the right and left masseter was the same However among the elevator muscles the masseters showed the largest differences in the degree of activity between the right and the left muscle in the single subject (cf Table 13) Differences in the pattern of activity between the right and the left side appeared from the very first stroke and were accentuated during the following strokes (Fig 39 A)

The activity during chewing in *the internal pterygoid muscles* has not been studied previously During free mandibular movements (*Moyers* 1950 *Carlsoo* 1952 *Zenker* and *Zenker* 1955 and *Surila* et al 1960) there was activity during protrusion and in the contralateral muscle during translation In view of these findings the early contralateral activity observed during unilateral chewing is assumed to direct and to stabilize the mandible toward the side of chewing while the ipsilateral muscle takes part in the deformation of the bolus During natural chewing the internal pterygoid muscles were the first elevators to be innervated during the closing movement the onset of their activity was 40 msec ahead of the anterior temporal muscle

Electromyographic studies of *the external pterygoid muscles* have shown activity during opening protrusion contralateral rotation and to some extent during biting in the intercuspal position (*Moyers* 1950 *Zenker* and *Zenker* 1955 *Carlsoo* 1956 b *Woelfel* et al 1960 *Ekholm* and *Surila* 1960) My experiments on chewing have shown that the external pterygoid mu cles are activated both in the opening and closing movement the largest activity being displayed during opening In keeping with *Hickey* et al s (1963) observations I found predominance of the contralateral muscle during unilateral chewing they did not mention the econdary activity observed simultaneously with the action of the elevator muscles The question is whether this secondary activity is in fact activity conducted from the elevator

muscles *Ekholm* and *Siirila* (1960) compared recordings with bipolar and concentric needle electrodes from the external pterygoid muscle and found that the concentric electrode picked up activity from the temporal and masseter muscles However, as judged from the large fluctuations of the base line their bipolar recordings were distorted because the impedance of the electrode was high as compared to the input impedance of the amplifier This in conjunction with the smaller amplitudes obtained with bipolar needle recordings probably accounts for the difference between the recordings from the two types of electrodes It seems unlikely that the secondary activity in my recordings was conducted activity from the elevator muscles the rejection of common voltage of the recording system was high as was the amplitude of the potentials (in Fig 35 42 B and 43 B 200–600 μV) Another source of error is a direct recording from the internal pterygoid because of the intimate anatomical relationship between the internal and external pterygoid muscles at their origins (see Fig. 3 B) The recordings of chewing movements presented by *Carlsoo* (1956 b) were most likely affected by this error To avoid this interference I have recorded from the external pterygoid muscle halfway between its origin and insertion (Fig. 3 B)

An excess of activity in one external pterygoid muscle results in a rotation towards the side with least activity Hence their unilateral patterns indicate a movement towards the chewing side during opening and an opposite movement in the final phase of the stroke this pattern of movement is in accordance with the findings in studies of the masticatory movements of the mandible (*Hildebrandt* 1931 *Koivumaa* 1961 *Beyron* 1964 *Ahlgren* 1966) The secondary activity in the external pterygoid muscles is therefore interpreted as a means of directing the mandible firstly toward the chewing side (contralateral muscle) and then toward the intercuspal position (ipsilateral muscle) rather than as a stabilizing action to prevent backward displacement of the condyles (*Carlsoo* 1956 b)

As to *the digastric muscles* there was a predominance of the contralateral muscle in one-sided mastication and the assumption that they are most active in ipsilateral movements (*Zenker* and *Zenker* 1955 *Eschler* 1958) was thus not confirmed Nor were the findings of *Carlsoo* (1956 a) and *Kawamura* (1957 a) confirmed that the digastric and elevator muscles differ in that the digastric muscles are less relaxed between the primary actions In my experiments one of the elevator muscles the internal pterygoid muscle revealed distinct intermediary activity (Fig. 34 A 2 and 42 B) moreover the difference between the intermediary activity in the digastric and that in the temporal and masseter muscles (Fig. 42 B and 43 B) to a large extent was caused by the different electrodes the needle electrodes in the digastric muscles gave three times larger amplitudes of the potentials than the

surface electrodes used for recording from the temporal and masseter muscles

According to *Carlsoo* (1956 a) the intermediary activity in the suprahyoid (digastric and mylohyoid) muscles during chewing serves to stabilize and guide the closing movement and therefore disappears almost entirely in the final stage when the jaw is directed by the teeth My recordings of tooth contact rather seem to indicate that the onset of the primary action of the digastric muscles occurs when tooth contact is established or even sooner However the opening activity during chewing differs from that during free movements when the onset of activity in the suprahyoid muscles occurs after the teeth are separated *(Zenker* and *Zenker* 1955 and *Garnick* and *Ramfjord* 1962)

With one exception previous conclusions with respect to the innervation of the *mylohyoid muscles* were based on the integrated pattern of the suprahyoid muscles as a whole Only *Zenker* and *Zenker* (1955) studied the mylohyoid muscles separately by intramuscular recording During free mandibular movements they observed a pattern very similar to that of the digastric muscles My experiments on chewing indicated a separate pattern for each of the two pair of suprahyoid muscles the most obvious difference being a time dispersal at the occurrence of maximal activity with the mylohyoid muscle as the leading one

In *Tulley s* (1953) recordings as in mine from *the orbicularis oris muscle* the largest activity during chewing occurred in the opening movement since he led off between one lead on the upper and one on the lower lip the separate pattern of each lip could not be evaluated I found that the maximal mean voltage in the lower lip was twice that in the upper

Tooth contact

Observations with an electrical contact between two antagonistic full crowns made *Jankelson* et al (1953) believe that tooth contact during mastication was negligible I found a high incidence of tooth contact in agreement with observations with cinematography cinefluorography electrical contact recorded directly or by telemetry in fully dentate subjects *(Hildebrandt* 1931 *Anderson* and *Picton* 1957 *Koivumaa* 1961 *Gillings* et al 1963 *Sheppard* and *Markus* 1962 *Graf* and *Zander* 1963 and 1964 *Beyron* 1964 *Scharer* and *Stallard* 1965) The sounds from the occlusion of the teeth have also been used to estimate the occurrence of tooth contact (*Brenman* and *Hattler* 1963 *Watt* 1963 *Brenman* and *Amsterdam* 1963) However sound is hardly suitable to study tooth contact during mastication because the comminution of the food produces sounds as well

Table 19

Comparison of the findings of Graf and Zander (1964) and of this study with respect to the relation in time between the electrical activity in the right anterior temporal muscle and tooth contact during natural chewing All figures (average ± SE) were referred to the onset of activity

Study	Number of subjects	Total duration of activity msec	Time to maximal activity msec	Make of tooth contact msec	Break of tooth contact msec	Location of contact
A	3	288±6	177±26	246±14	351±11	Molar
B	36	306±8	206±6	245±7	378±7	Incisor
t[1] of difference between A and B		1 8 p>0 05	1 1 p>0 20	<0 1	2 1 p<0 05	

A chewing of peanuts recording of contact was confined to the intercuspal position (data obtained from the diagrams of Graf and Zander 1964 Figs 6 7 and 8)

B chewing of apple recording of contact in the intercuspal position and in small lateral and protrusive movements i e less precisely than in A (data from this study with the time of maximal activity corrected for the 15 msec delay of the mean voltage)

[1] The t test was applied for the comparison

Anderson and *Picton* (1957) recorded contact in the balancing side during unilateral chewing and concluded that contact occurred only in the intercuspal position However their contact was adjusted to signal only in this position and my experiments indicate that contact occurred at different times from one section of the dental arches to another

As regards the relation between the duration of the contact interval and the total duration of the chewing cycle my findings confirm the observations of *Sheppard* and *Markus* (1962) that about 20 per cent of the time required for mastication was involved in possible tooth contacts

During natural chewing of apple molar contact was made 40 msec before and broken 50 msec before incisor contact (Table 17) A comparison with the time of make and break of contact in the intercuspal position (*Graf* and *Zander* 1964 referred to the onset of activity in the right anterior temporal m Table 19) shows that initial contact on the molars occurs in mandibular positions other than the intercuspal position and probably ceases when the mandible is in the intercuspal position This sequence of mandibular positions during chewing is consistent with a transverse slide in the closing phase of the chewing cycle (*Hildebrandt* 1931 *Koivumaa* 1961 *Beyron* 1964) and with a delay between premolar contact and full intercuspidation (*Scharer* and *Stallard* 1965)

Electrical and mechanical activity

When relating the electrical activity of muscle to the occurrence of tooth contact it is necessary to allow for the delay between electrical and mechanical activity and for the fact that tension persist for some time after the electrical activity has ceased In the muscles of mastication values of the delay between mechanical and electrical activity are not available In isometric voluntary contractions of the muscles of the arm and the leg made as rapidly as possible *Inman* et al (1952) observed a delay of 80 ± 20 msec between the maximum of the integrated electrical activity and the peak tension they stated that this delay was constant irrespective of muscle length and the same for various muscles (pectoralis major brachial biceps and triceps) *Desmedt* (1958 adductor pollicis m) found that tension in a twitch lasted for 300 msec and that peak tension was reached about 80 msec after the electrical stimulus in a tetanic contraction tension developed over more than 200 msec and it outlasted the electrical stimuli by about 150 msec The delay between maximal electrical activity and peak tension in the temporal muscle is probably about 100 msec because the chewing stroke is too short to attain the tetanic level In the diagrams of Fig 31 *I* the delay between electrical and mechanical activity causes the time course of the mechanical activity to be displaced to the right in relation to the electrical activity Therefore the maximal mechanical activity occurs somewhat later than the make of tooth contact The fact that the mechanical activity outlasts the electrical activity is consistent with the maintenance of tooth contact some time after the electrical activity has ceased (*Graf* and *Zander* 1964 and own experiments p 100) and makes it unlikely that tooth contact should cease before the activity in the anterior temporal muscles (*Scharer* and *Stallard* 1965 Fig 3)

The strong mechanical activity in the first part of the interval of contact and the time dispersal between the onset of contact in the different regions (cf Fig 42 A and 43 A) suggest a tilting of the mandible in the last phase of the closing movement The additional mechanical activity displayed simultaneously with contact in all regions could be responsible for the gliding between upper and lower teeth observed by *Hildebrandt* (1931) *Koivumaa* (1961) and *Beyron* (1964)

The later break of contact between the incisors than between the molars (cf Fig 42 A and 43 A) could result from either a sliding or a tilting movement of the mandible When the delay between electrical and mechanical activity is taken into consideration the action of the external pterygoid muscles probably occurred too late to accomplish a forward slide in the final phase of the interval of contact The early activation of the mylohyoid muscles could on the other hand account for a tilting since only their posterior ver

tically directed fibres can be assumed to take part in depressing the mandible *(Sicher* 1965)

Sherrington (1917) showed that pressure of weak or medium strength applied to the gums the teeth or the hard palate of a decerebrated cat resulted in an opening movement with a quick return to the closed posture when the stimulus was withdrawn. Since repetitive stimuli led to masticatory movements he concluded that the masticatory reflex depends on pressure stimuli exerted by the bolus Therefore stimulation of the pressure receptors of the periodontal membrane caused by the contact between the upper and the lower teeth has been considered an important link in the reciprocal innervation of the elevator and depressor muscles of the mandible *(Perry* 1956 *Jenkins* 1960 *Posselt* 1962) However the strong mechanical activity in the first part of the interval of contact and the absence of a systematic relation between the make of tooth contact and the time course of the activity in the right anterior temporal muscle (Table 18) indicate that the make of tooth contact does not abolish elevator activity It rather permits the increase in tension necessary to reduce the food particles to a suitable size for mixing with the saliva. The correlation between the occurrence of maximal activity in the temporal muscle and the onset of activity in the digastric muscles points to the tendon receptors and the muscle spindles as means of regulation. influenced by the tension developing in the closing movement they inhibit the elevators and facilitate the depressors

SWALLOWING

Swallowing was characterized by synergistic activation of all muscles but the degree of activity in the different muscles varied widely from subject to subject This is exemplified by the electromyograms obtained from two subjects during swallowing of saliva (Fig 44 A and B) and from one subject during swallowing of apple (Fig 44 C) The first subject swallowed with marked activity in all muscles and made tooth contact first in the incisor (9 a) and then in the molar regions (9 b and c) Activation of the lower lip (8 b) and the external pterygoid muscles (5) initiated swallowing The masseter muscle (3) was innervated later and for a shorter time than the other elevator muscles (1 2 and 4) The mylohyoid muscle (7) was active at the same time as the elevators while the strong action in the digastric muscle (6) occurred later

In the second subject (B) swallowing of saliva was performed with slight activity in the temporal (1 and 2) the masseter (3) and the lip (8) muscles and tooth contact (9) was absent The internal pterygoid muscles (4) were the only elevators with marked activity The difference between the activity in the elevators in this and the first subject (A) was not reflected in the degree of activity or in the coordination of the digastric and mylohyoid muscles (6 and 7)

The electromyograms from the third subject (C swallowing of apple) showed strong activity in the elevator muscles (1–4) slight activity in the lips (8) and there was tooth contact (9) As compared to the findings during natural chewing of apple (Fig 42 B) the antagonistic pattern of elevator and depressor muscles during chewing changed to the synergistic innervation of all muscles during swallowing In the digastric (6) and the mylohyoid (7) muscles the activity tended to occur in two (mylohyoid) and three bursts (digastric) In spite of unchanged activity in the temporal muscle incisor contact nearly broke for a short while (arrow) this was not an artefact due to displacement of the bands on the incisors

The anterior temporal and the mylohyoid muscles

Anterior temporal muscles The activity during swallowing in the right anterior temporal muscle of two subjects is exemplified in Fig 45 (recordings were obtained at three different sessions) Subject A showed wide variation in maximal mean voltage (50 µV–150 µV) and in the total duration of activity (800–1500 msec), the variation was less in subject B (mean voltage 60–90 µV, tot duration 700–900 msec) The fast recording allowed the exclusion of post swallowing activity (seen in A I a) when the total duration of activity was measured In the recordings obtained during swallowing a uniform indication of the reference point (onset of activity in the reference muscle) was secured by considering all recordings from the reference muscle together In cases with low and nearly constant activity during swallowing (Fig 46) the average level of the mean voltage was listed as the maximal mean voltage and the time parameters of the mean voltage were marked out by comparison with the electromyogram

The variance analysis of the data from the reference muscles was carried out separately for each type of swallowing During swallowing of saliva the variation of the activity in the right anterior temporal muscle between subjects was significant ($p < 0\ 001$) with respect to the total duration of activity as well as the maximal mean voltage and its time course The variation between the single experiments on the same subject was random Hence the large random variation exceeded the systematic differences caused by the position of the electrodes or by simultaneous use of needle electrodes

During swallowing of apple the total duration of the activity and the maximal mean voltage differed significantly between subjects and between single experiments on the same subject This was probably due to differences in the amount and the consistency of the food to be swallowed

The activity in the right anterior temporal muscle during swallowing was on the average accomplished in about one second the period of strong activity lasted 700 msec or about two thirds of the total duration (Fig 47 appendix Table XI) Swallowing of apple was characterized by slightly shorter duration and a greater intensity (925 ± 35 msec 113 ± 11 µV) than swallowing of saliva (1060 ± 40 msec 84 ± 10 µV)

In contrast to chewing the maximal mean voltage was positively correlated to the total duration of activity ($p < 0\ 001$) the total duration increasing with the degree of activity

The distribution of the total duration of activity was positive skew ($p < 0\ 01$) and leptokurtic ($0\ 01 < p < 0\ 05$) in 13 of 36 subjects the activity lasted for about one second or close to the average (1060 msec) 16 had a shorter and only 7 a longer duration (Fig 48 a 1) As regards the maximal

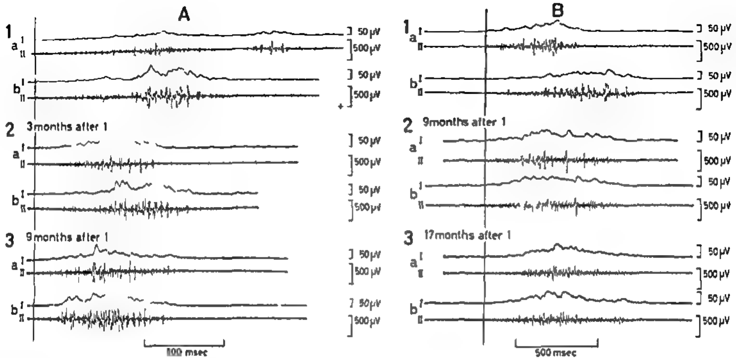


Fig 45

To illustrate the variability in electrical activity in the right anterior temporal muscle (*I* mean voltage *II* electromyogram) *during swallowing of saliva* The recordings were obtained in 3 different sessions (1 2 and 3) and in each session (a) represents the minimum and (b) the maximum of activity observed in a series of swallows

A Recordings obtained from a subject with large variation of the maximal mean voltage (50–150 µV) and of the total duration of of activity (800–1500 msec) Subject 2 23½ year old

B Recordings obtained from a subject with small variation of maximal mean voltage and of the total duration of activity (60–90 µV and 700–900 msec) Subject 9 25 years old

The vertical lines indicate the onset of activity (surface electrodes)

In the right anterior temporal muscle (reference m) the difference be tween the two types of swallowing was evaluated by comparing the average parameters In the left muscle this difference was tested during the variance analysis As on the right side there was a significant difference with respect to the time of maximum and the end of the period of strong activity The maximal mean voltages (saliva 79 ± 11 μV, apple 96 ± 11 μV) differed little Hence the single experiment from each type of swallowing did not reveal the slightly increased action when swallowing food as compared to swallowing of saliva

Mylohyoid muscles In view of the low activity displayed by the right anterior temporal muscle in some of the subjects (cf Fig 44 B), the right mylohyoid muscle was used as an additional reference in connection with the left mylohyoid the external and internal pterygoid and the digastric muscles The mylohyoid muscle was chosen as reference due to its insertion on the hyoid bone and its intimate connection with the movements of the tongue and larynx The activity in the mylohyoid muscle during swallowing (Fig 50) was usually preceded and succeeded by a short interval of low activity which was most pronounced in the post swallowing period (A 2 and 3 B 3 and 4) The position of the electrode strongly influenced the ampli tude of the interference pattern recorded during swallowing its effect on the time course of the activity was less pronounced (Fig 50 B)

The variance analysis of the findings in the right mylohyoid muscle gave about the same result as in the temporal except for the significant variation in maximal mean voltage in different experiments on the same subject this was most likely caused by changes in the position of the electrode (cf Fig 50 B) The total duration of activity during swallowing of saliva and apple (Fig 47 B appendix Table XI) was about 1100 msec or about the same as in the temporal muscle while the period of strong activity was shorter than in the temporal (550 ± 40 msec as compared to 675 ± 40 msec)

Also the action of the mylohyoid muscles tended to be briefer and stron ger when apple than when saliva was being swallowed (apple 1050 ± 30 msec and 227 ± [illegible] μV saliva 1125 ± 30 msec and 211 ± [illegible] μV) Signifi cant differences were only observed as regards the onset of the period of strong activity and the time of maximal activity ($p < 0.001$) On the average the right and left mylohyoid muscles were innervated symmetrically [illegible] both types of swallowing (cf appendix Tables XI and XII) The distributions of the total duration and maximal mean voltage were normal and the activi ty in the mylohyoid muscle (maximal mean voltage) was greater during swal lowing than during natural chewing (Fig 48)

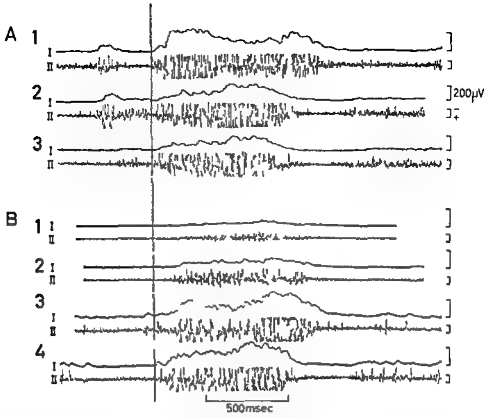


Fig 50

To illustrate the variability in the electrical activity and the influence of electrode positioning in recordings from the right mylohyoid muscle during swallowing of saliva (*I* mean voltage *II* electromyogram)

A Three subsequent recordings with the same electrode position maximal mean voltage *1* 246 µV *2* 258 µV *3* 222 µV

B Four recordings obtained 14 days after A 1 and 2 obtained before the final adjustment of the electrode to maximal response (3 and 4) Maximal mean voltages *1* 62 µV *2* 106 µV *3* and *4* 255 µV

Note that the activity in the mylohyoid muscle was larger before and after swallowing than in the temporal muscle (Fig 45) mainly because the former was led off by needle electrodes note also the short interval of low activity in the post swallowing period (A 2 and 3 B 3 and 4) Subject 36 25 years old

The vertical line indicates the onset of activity (needle electrodes)

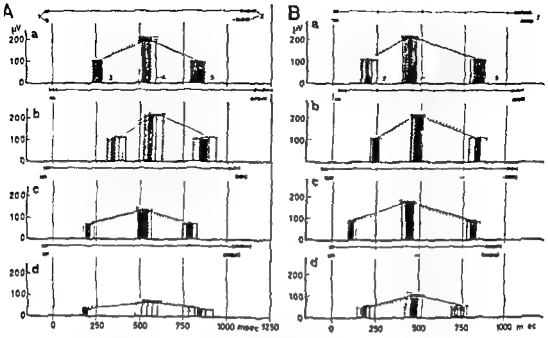


Fig 51

Comparison of findings in different test muscles during swallowing of saliva (A) *and apple* (B) *in recordings with the right anterior temporal* (full lines) *and the right mylohyoid* (stipled lines) *as reference muscle* (average findings in 36 subjects)

Time zero corresponds to the onset of activity in the right anterior temporal muscle the data obtained with the right mylohyoid as reference muscle were referred to a point 32 msec before time zero (average delay between the onset of activity in the mylohyoid and the temporal muscle was 32 ± 8 msec)

1 onset of activity *2* cessation of activity *3* time of 50 % max mean voltage *4* time of max mean voltage *5* time of 50 % decline from max mean voltage The uncertainty (average ± SE) of the time data is indicated by the shaded areas that of the maximal mean voltage by the largest distance between the horizontal lines on column *4*

The test muscles in the two series of experiments were the left mylohyoid (a) the digastric (b) the internal (c) and the external (d) pterygoid muscles

Suitability of the reference muscles The time and amplitude data obtained from the left mylohyoid the external and internal pterygoid and the digastric muscles were the same in both types of swallowing independent of whether the right anterior temporal or the right mylohyoid served as reference muscle (Fig 51) Only the results obtained with the right anterior temporal as reference muscle are discussed below The action of this muscle varied at random from experiment to experiment on the same subject and the activity in the various test muscles during swallowing was referred to its average pattern Differences in time might have been influenced by the low activity observed in some subjects Therefore differences at the onset of activity were re-examined (1) after 4 subjects with low level patterns in the right anterior temporal muscle had been omitted and (2) in 19 subjects with a rather constant maximal mean voltage of 50 μV or more in this muscle (appendix Table XIII)

Elevator muscles

(Fig 52 1–4 appendix Tables XIII and XIV)

During *swallowing of saliva* the periods of strong activity in the elevator muscles coincided (Fig 52 A) The onset of activity was synchronous in the two parts of the temporal muscle the masseter muscles were activated 41 ± 15 msec later and the internal pterygoid muscles 43 ± 15 msec before the reference muscle These differences in timing were also obtained when four subjects with low activity in the reference muscle were disregarded and when only data from the nineteen subjects were averaged whose activity in the reference muscle was more than 50 μV

During *swallowing of apple* (Fig 52 B) the coordination of the elevator muscles was as during swallowing of saliva but the activity in the masseter muscles was 60 per cent higher (saliva 60 ± 9 μV apple 97 ± 12 μV) and that in the internal pterygoid muscles 25 per cent higher (saliva 142 ± 6 μV apple 178 ± 7 μV)

The distribution of the maximal mean voltage in the posterior temporal muscle (Fig 53 a_1) showed the same moderate positive skewness ($0.01 < p < 0.05$) as in the reference muscle (Fig 48 A 2) In the masseter muscles the distribution of the maximal mean voltage was positive skew and leptokurtic ($p < 0.01$) 15 of the 36 subjects had a low amplitude in the masseter muscles during swallowing (less than 25 μV Fig 53 b_1) The distribution of the maximal mean voltage in the internal pterygoid muscles was normal (Fig 53 b_1) The activity in the elevator muscles was stronger during natural chewing (Fig 53 a b and c) than during swallowing

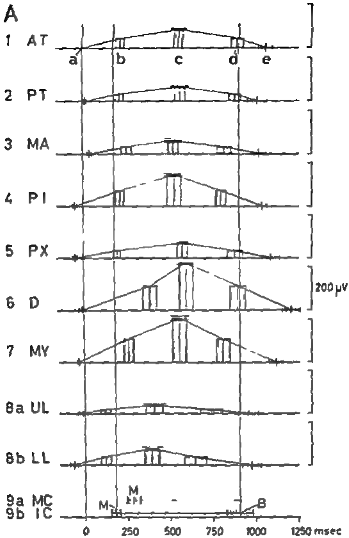


Fig 52

Average electrical activity in the elevator (1–4) depressor (5–7) and lip muscles (8) and the average time of make and break of molar (9a) and incisor (9b) contact during swallowing of saliva (A) and apple (B) (1–8 36 subjects 9a 5 subjects 9b 23 subjects) *a* onset of activity *b* time of 50 % max mean voltage *c* time of max mean voltage *d* time of 50 % decline from max mean voltage *e* cessation of activity

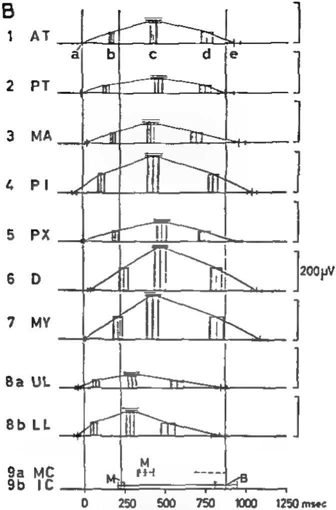


Fig 52 continued

The uncertainty (average ± SE) of the time data is indicated by the shaded areas that of the maximal mean voltage by the largest distance between the horizontal lines on column *c* The vertical line *0* indicates the onset of activity in the right anterior temporal muscle (time zero) the other vertical lines indicate the make (M) and break (B) of incisor contact M indicates the make of molar contact

AT right ant temp PT post temp MA masseter PI int. pterygoid PX ext pterygoid D digastric MY mylohyoid UL upper lip LL lower lip MC molar contact IC incisor contact

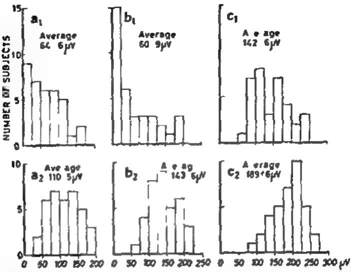


Fig 53

The distributions of the maximal mean voltages in the posterior temporal (a) *the masseter* (b) *and the internal pterygoid* (c) *muscles during swallowing of saliva* (1) *and chewing of apple* (2) *in 36 subjects* Note that the activity in all muscles was larger during chewing than during swallowing

Depressor muscles

(Fig. 52 5–7 appendix Tables XIII and XV)

During *swallowing of saliva* (Fig 52 A) the onset of activity in the external pterygoid muscles preceded the reference muscle by 45 ± 13 msec other wise the action of the two muscles coincided The digastric muscles were innervated in time with the right anterior temporal muscle but their strong action started later (dig 385 ± 40 msec right ant temp 220 ± 20 msec) Finally the mylohyoid muscles were activated 32 ± 10 msec before the tem poral muscle but in the further course of swallowing the two muscles were innervated simultaneously In subjects with strong activity in the reference muscle the time of onset of activity in this and the mylohyoid muscles did not differ significantly The strong activity in the mylohyoid muscles occurred 125 ± 40 msec earlier than in the digastric muscles This difference was found also with the right mylohyoid muscle as reference (100 ± 25 msec) The first part of the activity in the mylohyoid muscle corresponds to movements of the tongue in the oral phase the action of the digastric muscle initiates the pharyngeal phase by elevating the hyoid bone and the larynx Hence the peak of activity in these two muscles corresponds to the transfer of saliva from the oral cavity into the pharynx Their sustained action keeps the tongue the hyoid bone and the larynx elevated until the pharyngeal constrictors have brought the saliva into the oesophagus

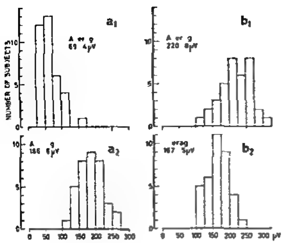


Fig 54

The distributions of the maximal mean voltages in the external pterygoid (a) and in the digastric (b) muscles during swallowing of saliva (1) and chewing of apple (2) in 36 subjects Note that the external pterygoid muscles were more active during chewing than during swallowing the reverse was the case in the digastric muscles

The activity in the external pterygoid muscles was slightly stronger during *swallowing of apple* (98 ± 8 µV Fig 52 B) than during swallowing of saliva (69 ± 4 µV) the maximal mean voltage in the digastric and the mylohyoid muscles was the same in both types of swallowing The external pterygoid and the mylohyoid muscles were innervated simultaneously with the reference muscle the digastric muscles were about 50 msec delayed ($p < 0\,005$) The onset of strong activity in the digastric and mylohyoid muscles coincided whether referred to the right anterior temporal or to the right mylohyoid muscle

The distribution of the maximal mean voltage recorded in the external pterygoid muscles (Fig 54 a_1) showed a marked positive skewness ($p < 0\,01$) and indicated a moderate degree of activation the strongest innervation during swallowing was below the average from chewing (Fig 54 a) In the digastric muscles (Fig 54 b_1 and b) the reverse was the case the maximal mean voltage during swallowing (220 ± 8 µV) being larger than that observed during chewing (167 ± 5 µV)

Orbicularis oris muscles

(Fig 52 8a and 8b appendix Tables XIII and XVI)

During *swallowing of saliva* (Fig 52 A) the activity in the upper lip started at the same time as in the reference muscle the activity in the lower lip started 60–70 msec earlier ($p < 0\,001$) and the phase of strong activity occurred 150 msec earlier than the corresponding phase in the reference muscle ($p < 0\,005$) Finally the maximal mean voltage in the upper lip (36 ± 5 µV) was only half that in the lower (75 ± 9 µV)

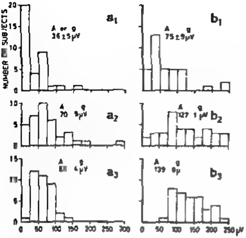


Fig 55

The distribution of the maximal mean voltages in the muscles of the upper (a) *and lower* (b) *lips during swallowing of saliva* (1) *and apple* (2) *and during chewing of apple* (3) Note the skew distribution of the activity in the upper lip during swallowing of saliva note also that the average activity in the lips was the same during swallowing and chewing of apple

During *swallowing of apple* (Fig 52 B) the activity in both lips was greater than when saliva was being swallowed the increase being 100 per cent in the muscles of the upper lip (36 to 70 μV) and 70 per cent in the lower (75 to 127 μV) Both in the muscles of the upper and lower lip the phase of strong activity occurred earlier than in the reference muscle ($p < 0\ 001$)

There was a marked positive skewness ($p < 0\ 01$) of the distribution of the activity in the lips during swallowing of saliva the maximal mean voltage in the upper lip was less than 25 μV in 20 of the 36 subjects in the lower lip 13 subjects had a maximal mean voltage between 25 and 50 μV 5 were below 25 μV 18 were between 50 and 250 μV (Fig 55 a_1 and b) Swallowing of apple was associated with a larger variation between subjects especially in the lower lip (Fig 55 a and b) The higher maximal mean voltage in the lower than in the upper lip during swallowing was observed in all subjects but one The distributions of the mean voltages during swallowing of apple resembled those during natural chewing (Fig 55 a_3 and b_3) in contrast to the other muscles the orbicularis oris serves the same purpose both during chewing and swallowing i e it seals the oral cavity

Tooth contact

The recordings of the make and break of tooth contact in the incisor region were often indistinct (Fig 56 A 1) probably due to small movements of the mandible before and after a period of firm contact

In the experiments the initial (M) and the final (B) signal of direct contact were used to indicate the duration of the interval of contact In most cases the molar recordings revealed distinct signals for make and break (Fig 56 A 2 and 3) Since the molar contacts were well adjusted crowns or inlays they were not as easily broken during small mandibular movements as the incisor contacts where precautions were taken to avoid that contact between the bands occurred earlier than between the upper and lower teeth

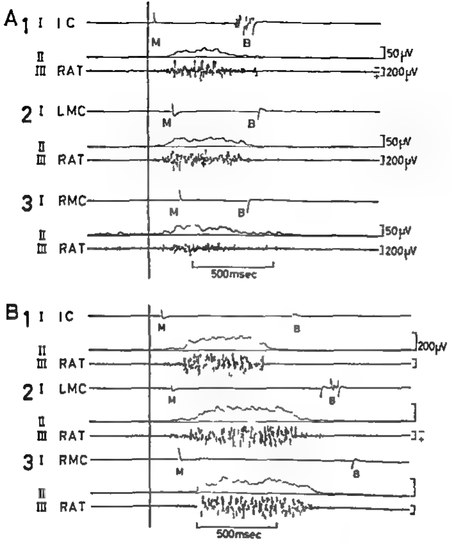


Fig 56

To illustrate the relation in time between tooth contact (*I* M Make B Break) *and the electrical activity in the right anterior temporal muscle* (RAT *II* mean voltage *III* electromyogram) *during swallowing of saliva*

A An example of moderate activity (about 50 µV 10 of 36 subjects) Note that the electrical activity outlasted the break of tooth contact and that incisor contact preceded molar contact (subject 20 23½ year old)

B An example of strong activity (150 µV [illegible] of 36 subjects) Note that tooth contact outlasted the electrical activity and that incisor contact preceded molar contact (subject 7 26½ year old)

The vertical lines indicate the onset of activity (surface electrodes IC incisor contact RMC right molar contact LMC left molar contact)

When the activity in the temporal muscle ceased gradually it usually out lasted the interval of contact (Fig 56 A) when it terminated abruptly, con tact was maintained for a short while after the cessation of the activity (Fig 56 B)

During swallowing of saliva incisor contact was present in 23 of 36 sub jects 7 subjects swallowed consistently without contact and in 6 contact occurred irregularly (Table 20) During swallowing of apple there was again contact in 23 of 36 subjects 12 swallowed without contact and in one the contact was irregular In the different subjects contact of the side regions

Table 20

Incidence of tooth contact during swallowing

Location of contact	Swallowing of	Number of subjects			
		With contact	Without contact	With irre gular contact	Total
Incisor region	Saliva	23	7	6	36
	Apple	23	12	1	36
Molar region	Saliva	5	3	1	9
	Apple	6	3	0	9

Table 21

Comparison of incisor and molar contacts

Location of contact	Incisor		Right molar		Left molar	
Swallowing of	Saliva	Apple	Saliva	Apple	Saliva	Apple
Subject no						
3*	+	+	/	Y	+	+
6	C	—	/	/	—	—
7	+	+	+	+	+	+
10		C	C	+	×	Y
15*	+	+	/	/	+	+
17	—	—	—	—	/	/
18	—	—	—	—	—	—
20*	+	+	+	+	+	Y
23*	+	+	+	+	+	+

* Subjects in whom the timing of incisor and molar contact could be evaluated
\+ Subjects with contact
— Subjects without contact
C Subjects with irregular contact
Y Not recorded

Table 22.

Average time dispersal between make and break of incisor and of molar contact during swallowing of saliva and apple Make of contact was referred to the onset of activity break to the cessation of activity in the right anterior temporal muscle (5 subjects cf Table 21)

Parameter	Average time dispersal			
	Saliva		Apple	
	M±SE	t	M±SE	t
Make of contact	−93±41 msec	2 3*	−154±48 msec	3 2 *
Break of contact	4±50 msec	0 1	19±94 msec	0 2

M average time dispersal SE standard error

$t = \frac{M}{SE}$ t* 0 01 < p < 0 05 t 0 001 < p < 0 01

Negative values indicate that incisor contact occurred before molar contact

both during swallowing of saliva and of apple was identical with the findings in the incisor region

In 21 subjects tooth contact (incisor region) was present both during swallowing of saliva and apple Differences either occurred in subjects in whom contact was irregular or when a change in the degree of activity could explain the different results Recordings from the incisors and molars differed only in one subject in each type of swallowing in both contact was irregular in the incisor region (Table 21) In five subjects with regular contact in the incisor and molar regions (cf Table 21) differences in the timing of the interval of contact could be estimated (Table 22) Make of contact was referred to the onset of activity in the right anterior temporal muscle break was referred to the cessation of activity in this muscle to reduce the uncertainty caused by the large variation of the total duration of activity in the single subject (cf Fig 56 B) In contrast to mastication contact during swallowing was made first in the incisor region and broken about simultaneously in the incisor and molar regions

During swallowing of saliva (Fig 57 A) the right anterior temporal muscle of subjects without contact (non–contact) exhibited a smaller maximal mean voltage and an earlier onset of strong activity (max mean volt 24 ± 8 μV time to strong act 103 ± 24 msec) than subjects with tooth contact (contact max mean volt 106 ± 12 μV time to strong act 240 ± 29 msec) The right anterior temporal muscle in subjects with irregular contact showed a maximal mean voltage (67 ± 16 μV) which was in between that of subjects with and without contact the standard errors of the time and

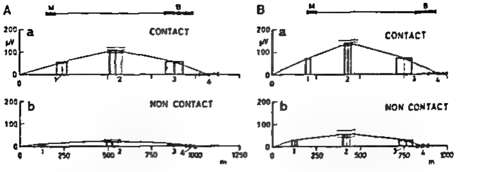


Fig 57

Average electrical activity in the right anterior temporal muscle during swallowing of saliva (A) and apple (B)

a subjects with contact (A and B 23 subjects) interval of contact indicated by the full line (above M make B break)

b subjects without contact (A 7 subjects B 12 subjects)

1 time of 50 % max mean voltage *2* time of max mean voltage *3* time of 50 % decline from max mean voltage *4* cessation of activity

The uncertainty (average ± SE) of time data is indicated by shaded areas that of the maximal mean voltage by the largest distance between the horizontal lines above column *2* Note that the maximal mean voltage was larger and strong activity occurred later in subjects with contact (a) than in subjects without contact (b) Time zero onset of activity in the right anterior temporal muscle (ref m)

amplitude data were 50–100 per cent larger than in subjects without contact When apple was being swallowed contact in only one subject behaved irregularly and findings in the subjects with and without contact (Fig 57 B) were similar to those during swallowing of saliva

The time course and the degree of activity in the right anterior temporal muscle during swallowing in the subjects with tooth contact (23) did not differ from the average findings in all subjects examined (36) Therefore a discussion of the timing of tooth contact can be based on the diagrams illustrating the average findings in all subjects (Fig 52) In both types of swallowing tooth contact relative to the period of strong activity in the elevator muscles was made and broken earlier than during chewing This is consistent with the absence of an obstacle which could delay the closing movement during swallowing The activation of the external pterygoid muscles simultaneously with the elevators at a time when contact was not yet established suggests a protrusion of the mandible in the initial phase of swallowing This is in keeping with the finding that contact on the incisors was made earlier than on the molars

In test muscles comparisons between subjects with contact without contact and with irregular contact were limited to the maximal mean voltages (Fig 58) In addition to the anterior temporal only the masseter muscles

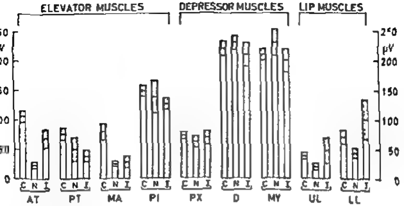


Fig 58

Average maximal mean voltages in the muscles of mastication during swallowing of saliva in 23 subjects with contact (C) *7 subjects without contact* (N) *and 6 subjects with irregular contact* (I) The shaded areas indicate the uncertainty (average ± SE) Note that subjects with tooth contact during swallowing had larger activity in the anterior temporal the masseter and the lip muscles than subjects without tooth contact

AT ant temp PT post temp MA masseter PI int pterygoid PX ext pterygoid D digastric MY mylohyoid UL upper lip LL lower lip

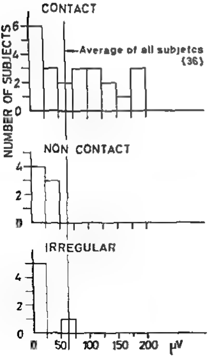


Fig 59

The distribution of the maximal mean voltage in the masseter muscles during swallowing of saliva in 23 subjects with contact 7 subjects without contact (non contact) *and in 6 subjects with irregular contact* (irregular) Note that 9 of 23 subjects with tooth contact during swallowing had a maximal mean voltage less than 50 µV

differed among elevators in subjects with and without contact (max mean volt. 'contact' 81 ± 13 μV, 'non-contact' 23 ± 4 μV) The incidence of contact increased with the degree of activity but 40 per cent of the subjects with a maximal mean voltage in the masseter muscles below 50 μV still showed contact (Fig. 59) In the depressor muscles the maximal mean voltages were the same regardless of whether there was contact or not The *maximal mean voltage in the muscles of the upper and lower lip was greater* in subjects with than in subjects without tooth contact ($p < 0.05$)

In subjects with irregular tooth contact the activity in the masseter muscles was similar to that of subjects without contact the action of the depressor and lip muscles was the same as in subjects with and without contact

Coordination in the individual subject

The distribution of activity in the individual subject was evaluated from the coefficients of correlation (r) of the maximal mean voltages in the different muscles (Table 23) In three groups of muscles the degree of activity was positively correlated i e varied in the same way

Table 23

Coefficients of correlation (r) between the maximal mean voltages in the muscles of mastication during sw- ing of saliva 36 subjects

Muscles	Ant temp	Post. temp	Masseter	Int. pterygoid	Ext pterygoid	Digastric	Mylohyoid	Upp lip
Post. temp	−0.71***							
Masseter[1]	−0.62 ** *−0.65 **	+0.51*** *+0.58 ***						
Int pterygoid	+0.32	+0.14	+0.08 *−0.07*					
Ext pterygoid[1]	−0.34* *−0.35**	−0.08 *−0.15*	−0.17 *−0.23*	−0.20 *+0.17*				
Digastric	−0.02	−0.01	−0.01 *+0.04*	−0.35*	+0.25 *+0.21*			
Mylohyoid	+0.08	+0.11	−0.04 *−0.08*	+0.35	+0.33 *−0.29*	+0.71***		
Upper lip[1]	−0.04 *−0.10*	−0.02 *−0.06*	−0.16 *−0.31*	−0.20 *0.21*	+0.28 *+0.26*	+0.14 *+0.15*	+0.19 *+0.16*	
Lower lip[1]	−0.02 *−0.03*	−0.06 *−0.01*	−0.07 *−0.20*	−0.23 *−0.20*	−0.09 *−0.03*	+0.10 *+0.04*	+0.17 *+0.12*	−0.[illegible] *+0.[illegible]*

[1] Distributions with a marked positive skewness ($p < 0.01$) the coefficients of correlation in italics obtained after logarithmic transformation of the skew distributions (see p 155)

I 0.01 < p < 0.05 I ** p < 0.001

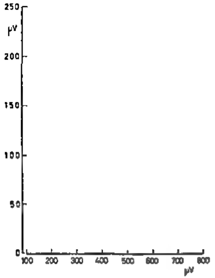


Fig 60

To illustrate the absence of a relationship between the maximal mean voltage in the lower lip muscles (ordinate) *and in the elevator muscles* (abscissa) *during swallowing of saliva* (36 subjects) The abscissa represents the sum of the maximal mean voltages in the four elevator muscles none of which were related to the mean voltage in the lower lip (cf Table 23)

1 The anterior and posterior parts of the temporal and the masseter muscles ($+0\,51 < r < +0\,71$ $p < 0\,001$)
2 The digastric and mylohyoid muscles ($r = +0\,71$ $p < 0\,001$)
3 The muscles of the upper and lower lip ($r = +0\,84$ $p < 0\,001$)

The activity in the internal and external pterygoid muscles was coordinated with both the anterior temporal (int $r = +0\,32$ $0\,05 < p < 0\,10$ ext $r = +0\,34$ $0\,01 < p < 0\,05$) the digastric (int $r = +0\,35$ $0\,01 < p < 0\,05$ ext $r = +0\,25$ $0\,10 < p < 0\,20$) and the mylohyoid muscles (int $r = +0\,35$ ext $r = +0\,33$ $0\,01 < p < 0\,05$) The activity of the three groups of muscles varied independently and the experiments showed no evidence of a specifically large action of the lips in subjects with low activity in all elevators (Fig 60)

Discussion

Swallowing mechanism in general

Magendie (1817) divided swallowing into an oral pharyngeal and oesophageal phase Radiological studies (*Mosher* 1927 *Johnstone* 1942 *Adran* and *Kemp* 1951 *Ramsay* et al 1955 *Cleall* 1965) have shown that the tongue and the larynx return to their pre swallowing positions during the initial part of the oesophageal phase The study presented in this report included muscles responsible for the elevation of the hyoid bone and the larynx and their activity corresponded in time to the oral and the pharyngeal phase

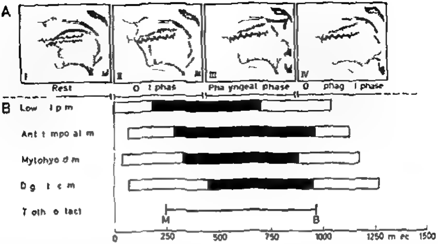


Fig 61

Duration of activity in the muscles of mastication (columns 36 subjects) *and of tooth contact* (M make B break 23 subjects) *during the three phases of swallowing* Black columns indicate the period of activity with more than 50 % of the maximal mean voltage *Time zero onset of activity in the muscles of the lower lip*

I Rest position

II Oral pha e anterior seal (lower lip m) elevation of the mandible (ant temporal m and make of tooth contact) action of the tongue (mylohyoid m)

III Pharyngeal phase elevation of the larynx (digastric m) maintenance of tooth *contact decreasing activity in the lower lip*

IV Oesophageal phase break of tooth contact cessation of activity in the lips Successive relaxation of the anterior temporal mylohyoid and digastric muscles Return of the mandible and the larynx to preswallowing positions

Swallowing mechanism redrawn from *Bjork* 1956

(Fig 61) The total duration of the electrical activity in the muscles involved in swallowing was 1 25 sec (onset of activity in the lips to cessation of activity in the digastric muscles) This fits with the duration of the oral and pharyngeal phase as determined by cinefluorography (*Ramsay* et al 1955 about one second *Cleall* 1965 1 5 sec)

Doty and *Bosma* (1956) studied the electrical activity during swallowing in 22 oral pharyngeal and laryngeal muscles in monkeys cats and dogs None of the three species had activity during swallowing in the temporal and masseter muscles whereas I found these muscles to be active in my 36 subjects However the maximal mean voltage was below 25 μV in the anterior temporal muscles in 8 subjects and in the masseter muscles in 15 subjects The digastric muscles were active only in the monkey and the activity was much smaller than during chewing In dog denervation of the mylohyoid muscles but not of the digastric muscles interfered with the pharyngeal phase of swallowing

activity in the digastric muscles in humans indicates therefore a difference from the other species examined

Rix (1946) has drawn attention to the possible importance of deglutition for the etiology of malocclusion He distinguished between (1) a normal basical swallow involving tooth contact but no particular activity in the lips and (2) an atypical or infantile swallow performed with the teeth apart and characterized by a spread of the tongue between the upper and lower teeth and increased tension of the lip and cheek muscles In patients with atypical swallowing malocclusion was found twice as often as in subjects with normal swallowing Based on the difference between the activity in the lips *Gwynne Evans* (1954) introduced the concept of omatic (tooth contact) and visceral (teeth apart) deglutition a classification accepted by most textbooks of oral physiology and orthodontics (*Saltzman* 1950 *Jenkins* 1960, *Schwartz* 1961 *Posselt* 1962) In subjects with extreme maxillary overjet *Bjork* (1958 a) distinguished a type of swallowing with tooth contact and the lower lip intruding between the upper and lower incisors *Strang* and *Thompson* (1958) included in *Rix s* (1946) atypical pattern a strong action of the elevator muscles in addition to the intense activity in the lips Finally *Silverman* (1961) only considered the teeth apart swallow associated with tongue thrust to be significant when the lips remained passive

In my group of adults the activity in the lip and elevator muscles as well as in the suprahyoid (digastric and mylohyoid) muscles varied independently during deglutition This may explain why the patterns of *Rix* (1946) and *Gwynne Evans* (1954) could not serve to classify the activity in the lips and the elevator muscles observed in previous electromyographic studies of swallowing (*Tulley* 1953 *Baril* and *Moyers* 1960)

Swallowing patterns in children

The incidence of tooth contact in my group of 36 adults (65 per cent with contact) agreed with that observed in children (*Rix* 1946 *Backlund* 1963 *Lieb* 1964 *Cleall* 1965) On the other hand in terms of non palpable activity in the masseter intense action of the lips and protrusion of the tongue *Fletcher* et al (1961) reported a decreasing number of atypical (visceral) swallowers from the age of 6 years (50 per cent) to 18 years (20 per cent) This estimate is however affected by the following uncertainties (a) the incidence of tongue thrust in 6–7 years old children is apt to be overestimated because of the temporary open bite (b) the high coincidence of strong lip action and tongue thrust is inconsistent with their alternating occurrence (*Subtelney* and *Subtelney* 1962) (c) longitudinal cinematographic studies (*Tulley* 1962) indicated a remarkable stability of the patterns of swallowing

from 6 years of age to adulthood (d) among 1500 eleven year-old children *Tulley* (1964) found only 40 with strong lip action during swallowing and protrusion of the tongue during articulation of S Electromyographic studies of deglutition in children are few and qualitative *Baril* and *Moyers* (1960) recorded bilaterally from the temporal, the lip and the buccinator muscles in 24 thumb or finger sucking children Ten children had atypical patterns of swallowing, eight normal and six could not be classified There was no evidence of a relation between the electromyographic patterns the thumb habit or the skeletal and dental aspects of the malocclusions *Baril* and *Moyers* (1960) only rarely found a delay between the activity in the lips and the temporal muscles In my experiments the muscles of the lower lip were activated 70 msec before the temporal muscle However with the recording speed used by *Baril* and *Moyers* (1960 6 cm per sec) this time difference corresponds to 3 mm of recording and could hardly be discriminated The time dispersal between the phase of strong activity in the lower lip and in the temporal muscle was more pronounced than at the onset of activity (150 msec 9 mm of recording at 6 cm per sec) but *Baril* and *Moyers* (1960) did not study the time course of the activity The early activation of the lips observed in my experiments is in keeping with the finding that the lips were most closed (i e most active) during the initial phase of swallowing (*Cleall* 1965)

In 11 males and 9 females 14–20 years old (with excellent dental occlusion) *Rosenblum* (1963) evaluated the activity in the area of the modiolus in terms of duration and intensity by cinematography of contour lines of the facial surface The degree of modiolus activity was described in arbitrary units (0–3) In agreement with the electromyographic findings the average activity of 0 5 indicated that the action of the lips during swallowing was slight The duration of modiolus activity (460 ± 70 msec *Rosenblum* 1963) was similar to the duration of the period of strong activity in the upper and lower lip (*610 ± 35 msec and 535 ± 35 msec respectively cf Fig* 52 8 a and 8 b)

Apparently the variation of the activity in the lip and elevator muscles during swallowing observed in the present study of adults was close to that observed previously in children however further investigations are required to decide definitely whether or not a change takes place during adolescence

Swallowing patterns in adults

In an electromyographic study of 40 dental students *Tulley* (1953) observed 15 with a normal pattern 10 with an atypical predominance of the lips and 15 innervated the lips as much as the masseter muscles This classifica

Table 24

Comparison of the findings of Graf and Zander (1964) and of this study [illegible] relation in time between the electrical activity in the right anterior temporal [illegible] contact during swallowing of food All figures (average ± SE) were [illegible] of activity

Study	Number of subjects	Total duration of activity msec	Time to maximal activity msec	Time to make of tooth contact msec	Time to break of tooth contact msec	[illegible]
A	3	488±61	252±31	185±29	587±70	[illegible]
B	23	944±54	415±21	224±21	874±60	[illegible]
t[1] of difference between A and B		5 6 p < 0 001	4 4 p < 0 001	1 1 p > 0 20	3 1 p < 0 01	–

A Swallowing of peanuts recording of contact was confined to the intercuspal [illegible] (data obtained from the diagrams of Graf and Zander 1964 Figs 9 10 and 11)
B Swallowing of apple recording of contact in the intercuspal position and [illegible] and protrusive movements i e less precisely than in A (data from this study [illegible] of maximal activity corrected for the 15 msec delay of the mean voltag.)
[1] The t test was applied for the comparison

tion was based on a qualitative comparison of the electromyograms from the two muscles the number of subjects in each group is roughly in accordance with the absence of correlation between the activity in the lip and masseter muscles during swallowing (Table 23) *Findlay* and *Kilpatrick* (1960) studied the activity in the temporal and masseter muscles during swallowing they used bipolar surface electrodes In the 24 subjects (18–23 years of age) the total duration and amplitude of the activity varied significantly from subject to subject and my findings are in keeping with this observation The total duration of activity averaged 2020 ± 100 msec with a range from 930 to 3200 msec (ant. and post temporal and masseter m *Findlay* and *Kilpatrick* 1960) as compared to 1060 ± 40 msec and a range from 750 to 1750 msec in my experiments (right ant. temporal m) However the example given by *Findlay and Kilpatrick (1960 Fig 2)* does not fit with their average findings the total duration of activity in all muscles (16 electromyograms from two swallows) varied from 250 to 1550 msec with an average of 1040 msec and the activity in the anterior temporal muscles (4 electromyograms from two swallows) lasted from 1270 to 1550 msec with an average of 1360 msec The electromyograms presented by *Findlay* and *Kilpatrick* (1960) indicate that the period of strong activity in the anterior part of the temporal muscle lasted for a little less than one second i e close to the duration of this period in my experiments (700 msec)

Graf and *Zander* (1964) studied the relation in time between muscle activity (temporal and masseter muscles) and the occurrence of tooth contact (intercuspal position) during swallowing of peanuts As related to the onset of activity in the right anterior temporal muscle the total duration of activity the time to maximal activity in this muscle and the time to break of tooth contact were significantly shorter than in my experiments (Table 24) I can offer no explanation for these differences they cannot be due to differences in amplification (*Graf* and *Zander* 1964 1 mm = 33 µV, own experiments 1 mm = 20 µV) On the other hand, the absence of a difference in the time of make of tooth contact (*Graf* and *Zander* 1964 intercuspal position own study incisors) may be explained by the fact that contact occurred 154 ± 48 msec earlier on the incisors than in the side regions (cf Table 22)

Sagittal dental relationship during swallowing

The relation between the upper and lower teeth in the sagittal plane during swallowing has been a matter of dispute On the basis of wax records and electromyograms of the temporal muscles obtained with the mandible retruded *Posselt* (1958) rejected a terminal hinge movement during swallowing and by means of cinefluorography *Cleall* (1965) found that the mandible in the initial phase of swallowing moved upward and forward from its position at rest my findings are in keeping with these observations Contrary to *Posselt* (1958) and *Cleall* (1965) *Ramfjord* (1961 a and b) considered contact during swallowing to occur primarily in a centric relation (terminal hinge relation) That swallowing takes place with the mandible in a retruded position was also concluded from an X ray study of mandibular movements (*Kydd* and *Sander* 1961) and from records of tooth contact both in the intercuspal position and in a position of retruded contact (terminal hinge relation *Graf* and *Zander* 1963) *Ramfjord's* (1961 a and b) conclusions were recently supported by *Scharer* and *Stallard* (1965) they found in simultaneous recordings of tooth contact (intercuspal position and transverse and sagittal sliding movements) and of the activity in the anterior and posterior temporal muscles that tooth contact during swallowing mainly involved a sliding movement and that the intercuspal position was only attained for a short interval of time However no data are given for the relation in time between incisor and molar contact and their recordings indicate a shorter period of contact in the intercuspal position than observed by *Graf* and *Zarder* (1964) This may indicate that the contact applied by *Scharer* and *Stallard* (1965) in the intercuspal position was too critical and their conclusion as to the direction of the slide needs further confirmation

There was no evidence of a terminal hinge movement during swallowing in my experiments The timing of the incisor and molar contacts and the muscle coordination in the short period before tooth contact indicated that tooth contact was made firstly on the incisors and then—most likely after a small backward slide—on the molars The slight retrusion observed by *Kydd* and *Sander* (1961 0 71 ± 0 7 mm) might explain the time dispersal between incisor and molar contact. This interpretation is not invalidated by the coincidence in retrusion and maximal activity in the tongue and the palate Molar contact was made simultaneously with the onset of strong activity in the mylohyoid muscle and maximal activity in this muscle occurred 275 msec later (cf Fig 52 A) On account of the delay between electrical and mechanical activity (about 100 msec see p 109) the make of molar contact (maximal retrusion) may precede maximal activity in the tongue (as determined by radiographs) by at most 375 msec The procedure of recording used by *Kydd* and *Sander* (1961 5 frames per sec each exposed 1/30 sec) would not allow to detect a time dispersal of 200 msec or less This in addition to the uncertainty in the estimation of the time to maximal activity of the tongue and palate might have prevented *Kydd* and *Sander* (1961) from discriminating a time dispersal of 375 msec

The different findings as to the sagittal relation of the teeth during swallowing in *Graf* and *Zander s* (1963) and in my experiments are difficult to explain Possibly the relation between contact signals and swallowing was less certain in *Graf* and *Zander s* (1963) studies in which the time of swallowing was visualized in cinematographic recordings of movements of the lips In my experiments simultaneous recordings of muscle activity and tooth contact ensured that the contact signals in fact were associated with swallowing The lead of the incisor recordings at the make of contact and the simultaneous break of contact in all regions exclude a forward slide On the other hand the absence of signals from the steel bands on the incisors does not exclude that contact may have occurred in a position posteriorly to the intercuspal position (see p 61) In fact three subjects who swallowed without signals from the incisor contact (classified as non-contact) showed activity above the average in the posterior part of the temporal muscle but below the average in the anterior part suggesting a retrusion of the mandible However simultaneous recordings of molar and incisor contact obtained in two of these subjects did not show evidence of molar contact This makes it unlikely that the subjects whom I have classified as swallowing without contact, in fact made contact in a position posteriorly to the intercuspal position

POSTURE AND FULL EFFORT

Postural activity

In these experiments the activity was recorded with the mandible at rest and in the position with the earliest tooth contact during closure ("occlusal position"). *In both positions the activity was slight (2–5 per cent) relative* to that observed during chewing and swallowing (Fig. 62). Occasionally the posterior part of the temporal muscle was more active in the rest position than the anterior part (Fig. 63). The intramuscular records contained often large discrete action potentials (Fig. 62, 7). That they may rarely occur also in surface recordings is illustrated in Fig. 64 (right ant. temporal muscle), the left anterior temporal muscle displayed the usual pattern of surface recordings. The frequency of the discrete discharges was about the same at rest (14–18 per sec) and in the occlusal position (17–18 per sec). In the evaluation of the mean voltage these individual spikes were disregarded. The recordings in the reference muscle varied significantly from subject to subject in both mandibular positions ($p < 0.01$). Repeated recordings in the same

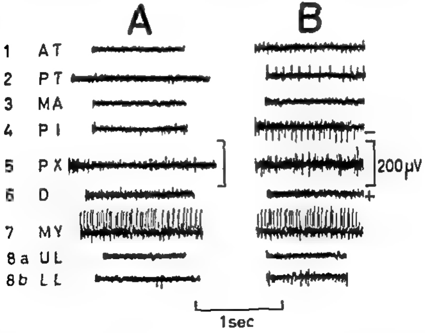


Fig. 62

To illustrate the postural activity in elevator (1–4), *depressor* (5–7) *and lip muscles* (8a, 8b) *with the mandible at rest* (A) *and in the position of earliest tooth contact during closure* ("occlusal position", B). Surface electrodes 1–3 and 8a and b, needle electrodes 4–7. AT ant. temp., PT post. temp., MA masseter, PI int. pterygoid, PX ext. pterygoid, D digastric, MY mylohyoid, UL upper lip, LL lower lip; subject 20, 23½ year old

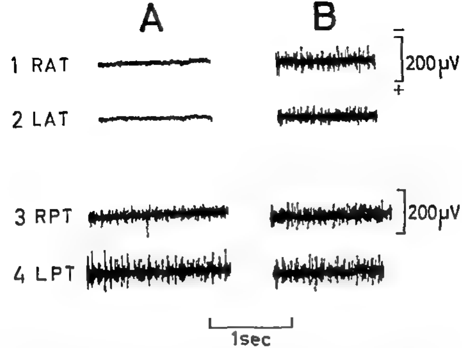


Fig 63

Example of predominance of the activity in the posterior parts of the temporal muscles (3 and 4) *as compared to the anterior parts* (1 and 2) *with the mandible at rest* (A) *and in the occlusal position* (B) RAT right ant temp LAT left ant temp RPT right post temp LPT left post temp surface electrodes subject 30 24^{1}/ year old

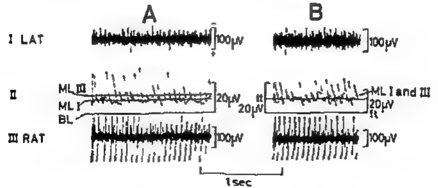


Fig 64

An example of the rare occurrence of discrete spike potentials when recording with surface electrodes from the right anterior temporal muscle (III and fat mean voltage trace of *II* RAT) *with the mandible at rest* (A) *and in the occlusal position* (B) The left anterior temporal muscle (*I* and thin mean voltage trace of *II* LAT) showed the usual pattern obtained with surface electrodes The spike potentials in *III* were disregarded in the evaluation of the mean voltage ML measuring lines ML *I* corresponds to the electromyogram in *I* ML *III* to that in *III* BL base line of the mean voltage Note the difference in the calibration of the thin (tt) and fat (ft) mean voltage trace in B subject 1 23 /$_2$ year old

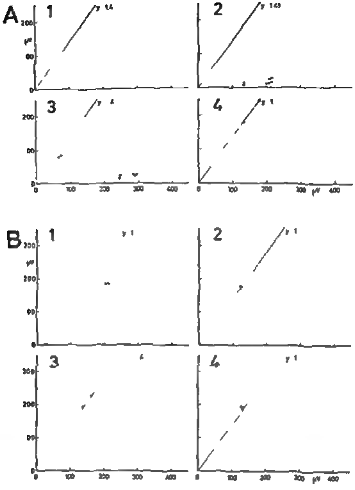


Fig 68

To illustrate the relation between the average level of the mean voltage during maximal bite (abscissa) *and the maximal mean voltage during swallowing of saliva* (A) *and during chewing of apple* (B) (ordinate) *in 36 subjects* *1* anterior temporal muscles *2* posterior temporal muscles *3* masseter muscles *4* internal pterygoid muscles

The line y = 1 41x represents the difference between the average level and the maximum of the mean voltage during maximal bite (41 ± 2 % right anterior temporal muscle 12 subjects) The points above the line indicate that the activity during swallowing (A) and chewing (B) exceeded the activity during maximal bite

recordings The activity during chewing came closer to the level obtained during maximal bite than the activity during swallowing (cf Fig 68 A and B) However even when allowance was made for the reduction in maximal mean voltage during chewing caused by overload of the amplifiers (see p 37) the activity during maximal bite represented the highest degree of activity observed in most of the 36 subjects (ant temp m in 31 post. temp m in 31 masseter m in 29 int pterygoid m in 30)

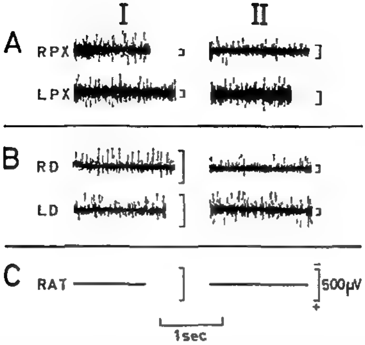


Fig 69

To illustrate the electrical activity in the external pterygoid (A RPX right LPX left) *and the digastric* (B RD right, LD left) *muscles and in the right anterior temporal muscle* (C RAT) *during maximal protrusion* (I) *and depression* (II) *of the mandible* Note that the external pterygoid muscles were most active during protrusion and that the digastric muscles were most active during depression note also the absence of activity in the temporal muscle Needle electrodes in A and B surface electrodes in C subject 28 29½ year old

Maximal protrusion and maximal opening

The activity during maximal protrusion and maximal opening was recorded only from the anterior temporal external pterygoid and digastric mu cles (Fig 69) The external pterygoid muscles were most active during protrusion (A I) the digastric muscles during depression of the mandible (B II) In both positions the activity in the right anterior temporal muscle was negligible (C I and II) The different patterns in the two depressor mu cles were confirmed statistically the activity in the external pterygoid muscles during maximal protrusion (194 ± 9 µV) exceeded that during maximal opening (144 ± 8 µV $p < 0.001$) In the digastric muscles the mean voltage during opening (190 ± 27 µV) was about four times that during maximal protrusion (51 ± 7 µV $p < 0.001$) In these experiments the determination of the mean voltage in the digastric muscles during maximal opening was associ

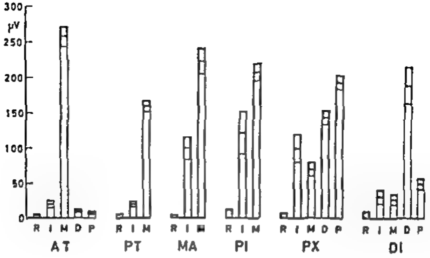


Fig 70

Average electrical activity in the elevator (AT PT MA and PI) *and depressor* (PX and DI) *muscles at rest and during full effort*

R activity with the mandible at rest (36 subjects)

I activity during maximal incisive bite (AT PT and MA 14 PI 7 PX and DI 9 subjects)

M activity during maximal bite in the intercuspal position (AT PT MA and PI 36 PX 20 and DI 17 subjects)

D activity during maximal depression (AT and PX 35 and DI 29 subjects)

P activity during maximal protrusion (AT and PX 35 and DI 29 subjects)

The shaded areas indicate the uncertainty (average ± SE) AT ant temp PT post temp MA masseter PI int. pterygoid PX ext pterygoid DI digastric)

ated with a high degree of uncertainty Later experiments showed that the maximal activity in the digastric muscles could be determined more exactly when opening was performed against resistance and with a smaller displacement of the mandible

In summary (Fig 70) during full effort the temporal muscles were active only in the intercuspal position During maximal incisive bite (imitating con ditions during biting off) the mandible was deprived of support from the tem poral muscles and fixed by the masseter and the internal and external ptery goid muscles The external pterygoid muscles were innervated strongly in all experiments but (in accordance with the direction of their fibres) most in tensively during protrusion Strong action of the digastric muscles was con fined to maximal opening

Discussion

Postural activity

According to *Shpuntoff* and *Shpuntoff* (1956) and *Jarabak* (1957) muscle activity was absent in the mandibular rest position Others have observed activity in the temporal muscles to the same degree in both the anterior and the posterior part (*Moyers* 1949 *Sirila* 1958) or with predominance in the posterior part (*Carlsoo* 1952 *Latif* 1957 *Kawamura* 1957 b) Expressed as a product of amplitude and frequency *Gopfert* and *Gopfert* (1955) found less activity in the masseter than in the temporal muscles they found no difference between the two parts of the temporal muscles My experiments indicated that the average degree of activity was the same in the anterior and posterior parts of the temporal muscle but the individual variation in the distribution of activity was considerable (cf Fig 62 and 63)

Contrary to *Carlsoo* (1952) and *Sirila* et al (1960) the experiments reported in this study demonstrated the presence of activity with the mandible at rest in the internal pterygoid muscles in all subjects except one The difference between *Carlsoo s* (1952) findings (activity in 1 of 7 subjects) and mine was due to the fact that his subjects were selected as having a minimum of activity with the mandible at rest in the report of *Sirila* et al (1960 *activity in 6 of 16 subjects) neither the gain nor examples of recordings were* given

Although the average results from the anterior temporal muscles indicated a small difference in activity between the rest and occlusal positions the mean voltage was unchanged in 13 of 36 subjects Moreover the activity in the masseter and internal pterygoid muscles was the same in both positions and determination of the rest position by the electromyogram seems rather uncertain (*Mullen* 1956 *Roberts* 1960 *Hickey* et al 1961 *Krajicek* et al 1961) That the activity in the temporal and masseter muscles decreases when the mandible is depressed beyond the rest position adds to this uncertainty (*Gopfert* and *Gopfert* 1954 *Garnick* and *Ramfjord* 1962)

The activity in the position at rest observed in the external pterygoid muscles is consistent with the findings of *Carlsoo* (1956 b) *Ekholm* and *Sirila* (1960) did not find this activity possibly on account of the localized pick up of their bipolar needle electrodes or the small amplitude obtained with this type of electrode

Full effort

The electromyograms obtained by *Latif* (1957) during maximal bite in the intercuspal position showed a uniform distribution of activity in the temporal

muscles while the recordings of *Greenfield* and *Wyke* (1956) indicated a predominance of the anterior part. *Woelfel* et al (1960 arbitrary values from 0–12 according to the spike amplitude) observed a uniform distribution of the activity in the temporal muscles significantly exceeding that in the masseter muscles Finally *Okun* (1960 the average of the 10 largest spikes) found a distribution of activity like that observed by *Greenfield* and *Wyke* (1956)

My results are in keeping with the recordings of *Greenfield* and *Wyke* (1956) and *Okun* (1960) although the difference between the two parts of the temporal muscle was less pronounced Since the electrical activity during maximal bite represented the upper limit of activity during chewing and swallowing, it seems justified to consider the mean voltage obtained during maximal bite as a measure of the maximal force which the elevator muscles can develop

In the case of maximal incisive bite *Latif* (1957) and *Woelfel* et al (1960) reported a 50–60 per cent reduction in activity in the temporal muscle as compared to maximal bite in the intercuspal position *MacDougall* and *Andrews* (1953) and *Greenfield* and *Wyke* (1956) reported a more substantial decrease (as judged from their electromyograms about 80 per cent) they reported reduced but distinct activity in the masseter muscles During maximal incisive bite (in contrast to maximal bite in the intercuspal position) I found in the temporal muscles almost a decrease to the resting level in the masseter muscles a decrease of 50 per cent in addition biting on the incisors was associated with strong action of the internal and external pterygoid muscles

The decrease in electrical activity in the masseter muscles (at a constant biting pressure) with increasing bite opening (*Garrett* et al 1964 see p 12) may be explained by the passive elastic force of the muscle carrying a larger part of the load on the muscle as its length increases The recordings of *Garrett* et al. (1964) were obtained with the mandible in protrusion when the anterior temporal muscles are nearly passive With increasing opening the position of the mandible changes the coronoid process moves downward which may cause a shift of some of the load to the anterior temporal muscles Hence to decide whether or not the electrical activity during biting with a given force in fact is reduced because of the opening, requires simultaneous recording from the temporal masseter and internal pterygoid muscles

The present recordings from the external pterygoid and the digastric muscles during protrusion and opening confirmed the qualitative observations of *Zenker* and *Zenker* (1955)

Chapter IV

THE ACTIVITY IN THE MUSCLES OF MASTICATION AS RELATED TO THE MORPHOLOGY OF THE FACIAL SKELETON

This section deals with the correlation between the activity in the muscles of mastication as determined by electromyography and the variation in the form and the size of the cranial base the jaws and the dental and alveolar arches The morphological measurements obtained from profile radiographs and dental casts were kindly placed at my disposal by *Solow* (1966)

ANALYSIS OF MORPHOLOGY AND OF CORRELATION

With a few exceptions *the roentgencephalometric analysis* was performed as outlined by *Bjork* (1958 b 1960 1963 a) using the reference points and lines shown in Fig 71 and 72

Measurements on dental casts (Fig 73) comprised overjet overbite and the sagittal occlusion of the molars taken according to the principles of *Seipel* (1946) Additional measurements on the casts were the width the length and the perimeter of the dental arches Finally spacing was determined as the difference between tooth size (sum of mesiodistal diameters of incisors canines and premolars) and the perimeter of the dental arches (in principle according to *Lundstrom* 1948 except that the first molars were omitted)

The variation in the morphology of the face and jaws of the 36 subjects examined in this study (Tables 28 29 and 30) was similar to that observed previously (*Bjork* 1947 1953 1955 *Lindegard* 1953 *Bjork* and *Palling* 1954 *Sarnas* 1959) except that the range of overjet overbite and the sagittal relation of the molars in my fully dentate subjects was less than in materials which include subjects with loss of teeth (*Bjork* 1947)

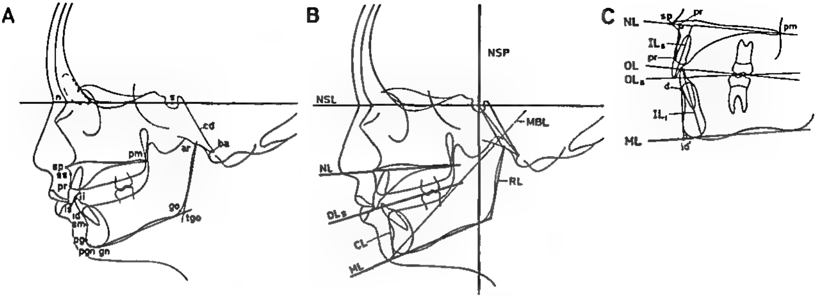


Fig 71

To illustrate and define the reference points and reference lines used in the roentgencephalometric analysis

A Reference points

Articulare (ar) intersection between the contour of the external cranial base and the dorsal contour of the condylar head
Basion (ba) the most inferior and posterior point of the clivus
Condylion (cd) the most posterior and superior point on the condylar head
Gnathion (gn) lowest point of the mandibular symphysis
Gonion (go) a point on the bony contour of the gonial angle determined by bisecting the angle ML/RL
Gonion tangent point (tgo) intersection between the mandibular line (ML) and the ramus line (RL)
Incision inferius (ii) mid point of the incisal edge of the most prominent lower central incisor
Incision superius (is) mid point of the incisal edge of the most prominent upper central incisor
Infradentale (id) highest and most prominent point on the lower alveolar arch
Nasion (n) most anterior point of the fronto-nasal suture
Pogonion (pg) most prominent point of the chin

Fig 71 continued

Prognathion (pgn) point on the bony contour of the chin determined by bisection of the angle between the mandibular line (ML) and the chin line (CL)
Prosthion (pr) lowest and most prominent point on the upper alveolar arch
Pterygomaxillare (pm) point representing the dorsal surface of the maxilla at the level of the nasal floor The point is located on the dorsal contour of the maxilla where this contour intersects that of the hard and soft palates
Sella (s) centre of the sella turcica The upper limit of the sella turcica is defined as the line joining the tuberculum sellae and the dorsum sellae
Spinal point (sp) apex of the anterior nasal spine
Subspinale (ss) (Down s A point) deepest point on the anterior contour of the upper alveolar arch
Supramentale (sm) (Down s B point) deepest point on the anterior contour of the lower alveolar arch

B Reference lines

Chin line (CL) tangent to the chin through the infradentale
Mandibular base line (MBL) line through the prognathion and the condylion
Mandibular line (ML) tangent to the lower border of the body of the mandible through the gnathion
Nasal line (NL) line through the spinal point and the pterygomaxillare
Nasion sella line (NSL) line joining the nasion to the sella
Nasion sella perpendicular (NSP) line through the sella and perpendicular to NSL
Ramus line (RL) tangent to the posterior border of the mandibular ramus and through the articulare
Occlusal line superior (OL) line through the incision superius and the distobuccal cusp of the upper first molar
Sella articulare line (s ar) represents the lateral part of the posterior cranial base
Sella basion line (s ba) denotes medial part of the posterior cranial base

C Reference points and reference lines in the dental region

Axis of lower incisor (IL_l) line from the incision inferius through the apex
Axis of the upper incisor (IL) line from the incision superius through the apex
Occlusal line inferior (OL_l) line through the incision inferius and the distobuccal cusp on the first lower molar
Projection of the infradentale on the mandibular line (id)
Projection of the prosthion on the nasal line (pr) Additional points and lines see A and B

Redrawn from *Björk* (1960) definitions were taken from *Björk* (1960) except MBL (*Sarnäs* 1959) and ba cd id and pr (*Solow* 1966)

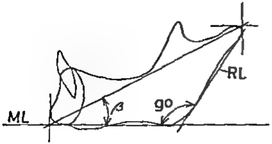


Fig 72

The form of the mandibular base in terms of the β angle (*Lindegård* 1953) and the gonial angle (go ML and RL see Fig 71 B) Redrawn from *Björk* (1960)

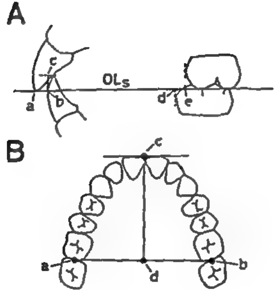


Fig 73

To illustrate and define the measurements on dental casts

A *Overjet* (a–b) the distance between the facial surfaces of the lower incisors and the most anterior point on the incisal edges of the upper central incisors in the upper occlusal plane (OL)
Overbite (b–c) the distance between the upper occlusal plane (OL_s) and the incisal edges of the lower central incisors
Sagittal occlusion of the molars (d–e) the distance between the mesial surfaces of the upper and lower first molars in the upper occlusal plane (OL_6 measured on both sides)

B *The width of the dental arch* (a–b) the distance between the contact point of the first and second molars on the right and left sides
The length of the dental arch (c–d) the distance between the most anterior point of the facial surfaces of the upper central incisors and a line connecting the contact points of the first and second molars on the right (a) and the left (b) sides the distance was measured in the occlusal plane Arch width and length were measured both in the upper and lower arch

Redrawn from *Solow* (1966)

In *the correlation analysis* the coefficients of correlation (r) were determined from

$$r = \frac{s_{xy}}{\sqrt{s_x^2 \cdot s_y^2}}$$

where s_x and s_y^2 are the variances and s_{xy} the covariance of the variates x and y The hypothesis that $r \neq 0$*) was tested by computing $t = \frac{r}{\sqrt{1-r}} \sqrt{f}$ with $f = n - 2$ degrees of freedom n being the size of the sample

Among the 35 distributions of *morphological data* used in the analysis of correlation (Tables 28 29 and 30) 22 showed no departure from normality A moderate skewness or kurtosis ($0.01 < p < 0.05$) was seen in eight distributions a marked deviation from normality was present in the following five (1) the inclination of the upper incisors (IL/NL) and (2) the sagittal relation of the first molars on the right side showed a negative skewness ($\sqrt{b_1}$ − 1 18 (1) and − 0 97 (2) 1 per cent limit − 0 92) (3) the sagittal relation of the first molars on the left side and (4) the spacing in the lower dental arch had platykurtic distributions (a 0 68 (3) and 0 70 (4) 1 per cent limit 0 72) finally (5) the distribution of the spacing in the upper dental arch was positive skew ($\sqrt{b_1}$ 1 37 1 per cent limit 0 92) Among the five morphological distributions with a marked departure from normality important correlations were only obtained in the case of the inclination of the upper incisors

With respect to the *electromyographic data* a number of distributions showed a significant departure from normality the most frequent deviation being a positive skewness (cf p 71) The correlations considered in the following involved 49 electromyographic distributions Among these distributions 27 showed no departure from normality 6 deviated moderately ($0.01 < p < 0.05$ 4 of these were positive skew 1 was platykurtic and 1 leptokurtic) and 16 distributions showed a pronounced positive skewness ($p < 0.01$ 1 was leptokurtic and 2 were platykurtic as well) In the case of a positive skewness a logarithmic transformation tended to normalize the distributions and reduced the influence of extremely large values on the coefficients of correlation Therefore the analysis of correlation was repeated with the corresponding logarithmic data of 11 of the 16 distributions with a marked positive skewness Among the 11 transformed distributions 7 showed no departure from normality (1) the maximal mean voltage of the secondary activity in the upper lip during chewing (2) the time to onset of strong activity in the right mylohyoid muscle (as reference muscle) during swallowing of saliva, (3) the maximal mean voltage in the external pterygoid muscles during swallowing of saliva, the postural activity in (4) the posterior temporal and (5) the digastric muscles (6) the maximal mean voltage in the lower lip during swallowing of saliva and (7) the maximal mean voltage in the upper lip during swallowing of apple Two distributions showed no skewness but remained leptokurtic (maximal mean voltage in the masseter muscles during swallowing of saliva) or showed a moderate leptokurtosis (maximal mean voltage in the upper lip during swallowing of saliva) after transformation one distribution changed from a marked positive to a moderate negative skewness (onset of strong activity in the right and left mylohyoid muscles during swallowing of saliva) the most extreme deviation from normality (postural activity in the lower lip $\sqrt{b_1} = 3.13$ a = 0 60) remained positive skew ($\sqrt{b_1} = 1.13$) but showed no kurtosis after the transforma

) In the text the probability that r arose by chance is denoted as follows r $0.01 < p < 0.05$ r** $0.001 < p < 0.01$ r*** $p < 0.001$

Table 28

Facial morphology of the 36 subjects examined

Region			Measurements[1]	Mean	Standard deviation	Range
Cranial base	Form	centrally	n-s ba	128 9	5 2	117.5 139 0
		laterally	n-s-ar	123 1	5 1	113 5 134 0
Mandibular base	Form	β-angle		29 3	3 7	23 0- 37 0
		gonial angle	RL/ML	119 7	7 5	105.5 134 5
	Size	length	pgn-cd mm	125 4	4 7	114 0-135 5
Cranial relationship of the jaws	Maxilla	sagittal	s n-ss	82 2	3 3	76 0- 90 0
		vertical	NSL/NL	7 4	2 8	2 0- 12.5
	Mandible	sagittal	s n pg	81 3	3 5	72.5- 90 5
			s n sm	79 4	3 3	71 0- 87 0
		vertical	NSL/ML	28 2	7 4	14 0- 47 0
			NSL/MBL	55.2	4 9	47 0- 68 0
Mutual relationship of the jaws		sagittal	ss-n pg	0 78	3 4	−4.5- 8 0
		vertical	NL/ML°	21 3	7 4	7 0- 36 5
Anterior face		height	n gn mm	126 1	7 1	106 5 143.5

[1] All measurements obtained from profile radiographs for definitions of reference points and lines see Fig. 71 in the case of the β-angle and gonial angle see Fig. 72.
angular measurements in degrees
mm linear measurements in millimetres
Negative value of the sagittal relationship of the upper and lower jaw (ss n pg) indicates that the pogonion (pg) is *anteriorly to the subspinale (ss)*

tion The coefficients of correlation given in the text are those obtained without logarithmic transformation in the tables the coefficients of correlation for the transformed data are given as well

The *two-dimensional distributions* corresponding to each of the coefficients of correlation were tested with respect to departure from normality according to *Hald* (1952) and *Solow* (1966) Among the 371 two-dimensional distributions examined 335 showed no deviation from normality 29 showed a moderate (0 01 < p < 0 05) and 7 a marked (0 001 < p < 0 01) departure Marked departures from normality of the two-dimensional distributions are indicated in the tables. Using the transformed data of the 11 electromyographic distributions mentioned above the number of two-dimensional distributions with a moderate departure from normality was reduced from 29 to 12 However the number of two-dimensional distributions with a marked departure was unchanged three distributions showed a marked departure before and no departure after transformation of the electromyographic data (Tables 40 42 and 44) on the other hand three distributions showed no departure before but a marked departure after transformation of the mean voltage (Tables 34 and 44) The remaining four two-dimensional distributions with a marked departure from normality had normal marginal distributions (Tables 32 37 and 39)

Table 29

Dento-alveolar morphology of the 36 subjects examined

Region			Measurements[1]	Mean	Standard deviation	Range
Dento-alveolar relationship[1]	sagittal	Maxill alveolar prognath	pr n ss	3 0	1 2	1 0- 6
		Maxill incisor inclin	IL_s/NL	110 3	5 5	93 0-118
		Mandib alveolar prognath	CL/ML	70 6	7 2	58 5 87
		Mandib incisor inclin	IL_i/ML	97 8	6 2	87 0-118
		Maxill /Mandib incisor inclin	IL_s/IL_i	126 9	11 1	106 5 143
	vertical	Maxill zone	OL_s/NL	7 6	3 6	−0 5 16
		Ant maxill alveolar height	pr pr mm	17 7	2 7	12.5 24
		Mandib zone	OL_i/ML	17 0	4 6	7 5- 25
		Ant mandib height	id id mm	33 1	3 2	27 5 43
Dental relationship[2]	Incisors	sagittal	overjet mm	3 2	1.2	1 0- 6
		vertical	overbite mm	3 4	1 5	0 0- 7
	1st molars	sagittal	right side mm	2.1	1 6	−2.0- 5
			left side mm	2.3	1 7	−2.5 5

[1] angular measurements in degrees
mm linear measurements in millimetres
[1] Measurements obtained from profile radiographs for definitions of reference points and lines s Fig. 71
[2] Measurements obtained from dental casts for definitions see Fig 73
Negative values of the maxillary zone (OL /NL) indicate that the reference lines NL and OL_s converg anteriorly
Negative values of the sagittal relationship of the first molars indicate that the mesial surface of the upp first molar is anteriorly to that of the lower first molar

Table 30

Morphology of the dental arches of the 36 subjects examined

Region	Measurements[1]	Mean mm	Standard deviation mm	Range mm
Maxilla	Arch length	36 7	2.0	31 5-41 5
	Arch width	51 0	2 6	46 5 55 0
	Perimeter	73 2	3 1	64 1 78 6
	Spacing[2]	0 8	2.5	−2.6- 7 9
Mandible	Arch length	32 2	2 1	27 0-36 5
	Arch width	47 0	2.7	42 0-53 5
	Perimeter	62 0	2 6	54 6-66 4
	Spacing[2]	−1 3	2 8	−5 9 7 3

[1] Measurements on dental casts for definitions see Fig 73 and p 151
[2] Negative values indicate crowding.

The analysis of correlation presumes normality of the distributions involved Therefore the correlations obtained with the non normal distributions represent an estimate rather than an exact measure of the association between morphology and function

The correlation between muscle activity and morphology is considered separately for elevator, depressor and lip muscles Moreover in four subjects the coordination of the mylohyoid and the lip muscles during swallowing was related to lip posture Aside from measurements of the width and the spacing of the dental arches the analysis was confined to vertical and sagittal facial dimensions

RESULTS

Elevator muscles

Chewing Subjects with strong activity in *the reference muscle* (right anterior temporal m) during natural chewing tended to have increased anterior face height (n gn r = + 0 29 0 05 < p < 0 10), most marked with respect to the anterior alveolar height of the maxilla (pr–pr r = +0 33*) Also the maximal mean voltage in *the posterior part of the temporal muscle* was positively correlated to the anterior alveolar height of the maxilla, in addi

Table 31

Coefficients of correlation (r) between
electrical activity the maximal mean voltage in the posterior temporal muscles during chewing.
facial morphology the anterior alveolar height of the maxilla upper incisor inclination and overbite

36 subjects

Facial morphology		Maximal mean voltage (µV)			
	Ref points or lines	Natural chewing		Unilateral chewing	
Region	(Fig 71)	Apple	Bread	Ipsilateral	Contralateral
Ant maxill alveolar height	pr pr mm	[illegible]39*	+0 45*	+0 34*	+0 38
Inclination of upper incisors	IL ∖NL	−0 34	−0 25	−0 39	−0 25
Overbite	mm For def see Fig 73	+0 44	+0 24	+0 47**	+0 27

angular measurement in degrees
mm linear measurements in millimetres
* 0 01 < p < 0 05 ** 0 001 < p < 0 01

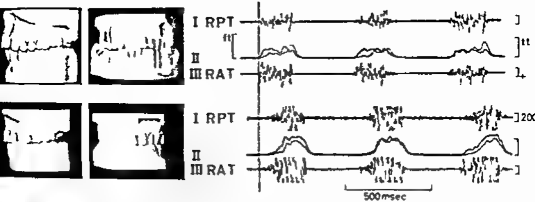


Fig 74

To illustrate that the degree of activity in the posterior temporal muscle during chewing was less in a subject with small overbite (A) *than in a subject with marked overbite* (B) In these subjects there was also a positive correlation between the overbite and the degree of activity in the anterior temporal muscle a correlation which did not appear in the material as a whole ($r = + 0.23$ $0.10 < p < 0.20$ 36 subjects)

A. Maximal mean voltage in the posterior temporal muscle 70 µV in the anterior 106 µV overbite 1 5 mm Subject 19 24 years old

B. Maximal mean voltage in the posterior temporal muscle 188 µV in the anterior 237 µV overbite 5 mm Subject 32 24 years old

Recordings obtained during natural chewing of apple *I* and thin mean voltage trace of *II* right posterior temporal muscle (RPT test m) *III* and fat mean voltage trace of *II* right anterior temporal muscle (RAT ref m) Note the difference in the calibration of the fat (ft) and the thin (tt) trace in A Vertical line indicates the onset of activity in the reference muscle (RAT) surface electrodes. For definition of overbite see Fig 73

tion strong activity in this muscle during chewing was associated with retroclination*) of the upper incisors and a large overbite (Table 31 Fig 74)

The maximal mean voltage in *the masseter muscle* did not show a relation to the morphological traits examined On the other hand an early activation of the masseter muscle (referred to the onset of activity in the right anterior temporal m) especially during unilateral chewing occurred in subjects with a flattened cranial base (Table 32 and Fig 75) The early activation of the masseter muscles was not related to the mutual relation of the jaws or the dental arches in the sagittal plane

Swallowing The maximal mean voltage in both parts of *the temporal muscle* during swallowing was correlated to the curvature of the cranial base particularly to that of its lateral parts strong activity being associated with a curved cranial base (Table 33)

*) The term retroclination is used when the angles IL /NL and IL/ML are small proclination is used when these angles are large

The analysis of correlation presumes normality of the distributions involved. Therefore the correlations obtained with the non normal distributions represent an estimate rather than an exact measure of the association between morphology and function.

The correlation between muscle activity and morphology is considered separately for elevator depressor and lip muscles. Moreover in four subjects the coordination of the mylohyoid and the lip muscles during swallowing was related to lip posture. Aside from measurements of the width and the spacing of the dental arches the analysis was confined to vertical and sagittal facial dimensions.

RESULTS

Elevator muscles

Chewing Subjects with strong activity in *the reference muscle* (right anterior temporal m.) during natural chewing tended to have increased anterior face height (n-gn. $r = -0.29$, $0.05 < p < 0.10$) most marked with respect to the anterior alveolar height of the maxilla (pr–pr $r = -0.33^*$). Also the maximal mean voltage in *the posterior part of the temporal muscle* was positively correlated to the anterior alveolar height of the maxilla in addi

Table 31

Coefficients of correlation (r) between
electrical activity the maximal mean voltage in the posterior temporal muscles during chewing.
facial morphology the anterior alveolar height of the maxilla, upper incisor inclination and overbite
[illegible] subjects

Facial morphology		Maximal mean voltage (µV)			
	Ref. points or lines (Fig. 71)	Natural chewing		Unilateral chewing	
Region		Apple	Bread	Ipsilateral	Contralateral
Ant. maxill. alveolar height	pr–pr mm	0.3[illegible]	−0.45	−0.34*	−0.38
Inclination of upper incisors	ILs NL	−0.34	−0.25	−0.3[illegible]*	−0.25
Overbite	mm For def. see Fig. 73	−0.[illegible]	−0.24	−0.47*	−0.27

° angular measurement in degrees
mm linear measurements in millimetres.
r* 0.01 p 0.05 r** 0.001 p 0.01

mal base and mandibular prognathism) and the sample's distributed mean voltage in the masseter muscle during swallowing is exemplified in Fig. 76 *a* and *b* The logarithmic plot (c and d) indicated that the correlations were not determined solely by the subjects with strong activity

Postural activity The activity in *the posterior temporal muscle* (Table 35) with the mandible at rest was large in subjects with prognathism of the upper jaw (s–n–ss r = +0 35 *) and of the apical base of the lower jaw (s–n–sm r = +0 32 0 05 < p < 0 10) No correlation was found between the postural activity in the posterior temporal muscle and the mutual relation of the jaws and the dental arches in the sagittal plane

Maximal bite (Table 36 Fig 77) The mean voltages in the elevator muscles during maximal bite in the intercuspal position were normally distributed Strong activity in *the anterior temporal muscles* was associated with a large anteriorly inclined mandible with an arch-shaped base (pgn–cd r = +0 38 *, NSL/ML r = −0 32 0 05 < p < 0 10 β-angle r = −0 32 0 05 < p < 0 10 gonial angle r = −0 33 *) The only maxillary data among these coefficients of correlation was the anterior alveolar height determined both directly (pr–pr ; r = −0 39 *) and by the inclination of the upper occlusal plane in relation to the nasal line (OL/NL r = −0 30 0 05 < p < 0 10)

Table 35

Coefficients of correlation (r) between

electrical activity the mean voltage in the posterior temporal muscles with the mandible at rest
facial morphology the cranial and mutual relationship of the jaws in the sagittal plane
36 subjects

Facial morphology		Mean voltage with the mandible at rest[1]	
Region	Ref points (Fig 71)	µV	log µV
Maxill prognath	s n ss	+0 35*	+0 35*
Mandibl prognath	s n pg	+0 22	+0 21
	s n sm	+0 32	+0 31
Mutual sagittal relationship of the jaws	ss n pg	+0 08	+0 06

[1] The distribution of the mean voltage (µV) was positive skew ($\sqrt{b_1}$ = 2 85 1 / limit 0 97) the transformed data (log µV) showed no departure from normality
angular measurements in degrees
r* 0 01<p<0 05

Table 36

Coefficients of correlation (r) between
electrical activity the average level of the mean voltage in the elevator muscles during maximal bite in the intercuspal position
facial morphology facial prognathism

36 subjects

Facial morphology Region	Ref points and lines (Fig. 71)	Mean voltage during maximal bite (µV) Ant temp m	Post temp m	Masseter m	Int pterygoid m
Cranial base	n-s ar	+0 14	+0 004	−0 10	−0 15
Mandibular base	β angle	+0 32	+0 15	+0 21	−0 01
	gonial angle	−0 33*	−0 26	−0 34*	+0 01
	pgn-cd mm	+0 38*	+0 19	+0 12	+0 23
Maxill prognath	s n-ss	+0 02	−0 14	+0 16	+0 31
inclin	NSL/NL	+0 02	+0 12	−0 01	−0 10
Mandib prognath	s-n pg	+0 31	+0 05	+0 35*	+0 36*
	s n-sm	+0 24	+0 03	+0 33*	+0 36
inclin	NSL/ML	−0 32	−0 14	−0 35*	−0 10
	NSL/MBL	−0 28	−0 12	−0 35*	−0 20
Mutual relationship of the jaws sagittal	ss-n pg	−0 26	−0 15	−0 25	−0 07
vertical	NL/ML	−0 35*	−0 22	−0 36*	−0 10
Ant face height	n gn mm	−0 01	−0 02	−0 25	−0 09
Maxill zone	OL_s/NL	0 30	+0 05	−0 15	+0 003
Ant maxill alveolar height	pr pr mm	−0 39*	−0 13	−0 45**	−0 18
Mandib zone	OL_i/ML	−0 16	−0 16	−0 21	−0 07
Ant mandib height	id id mm	−0 15	−0 05	−0 34*	−0 03
Overbite	mm For def see Fig. 73	+0 25	+0 40*	+0 30	+0 17

angular measurements in degrees β-angle and gonial angle see Fig 72
mm linear measurements in millimetres
r* 0 01 < p < 0 05 r** 0 001 < p < 0 01

The activity in *the posterior temporal muscle* during maximal bite as during chewing was correlated positively to the vertical relation of the incisors the overbite increasing with the degree of activity (r = +0 40) Although strong activity in the two parts of the temporal muscle coincided (r = +0 62 ***) the mean voltage in the posterior part showed only a tendency of a correlation to the form of the mandibular base

Subjects with strong activity in *the masseter muscles* were characterized by prognathism and anterior inclination of the mandible and a small gonial angle In contrast to swallowing the activity during maximal bite was corre

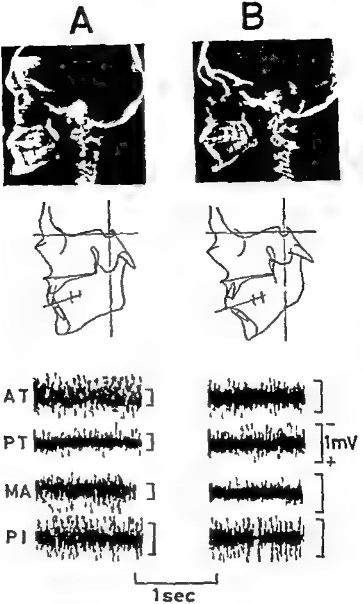


Fig 77

To illustrate the relation between the average level of the mean volta e in the eleva muscles during maximal bite and the form of the mandibular base and the prognath and inclination of the mandible Electromyograms obtained from the anterior (1 A and posterior (2 PT) temporal the masseter (3 MA) and the internal pterygoid (4 muscles during maximal bite in the intercuspal position

A Mean voltage during maximal bite AT 368 μV PT 219 μV MA 421 μV 190 μV
Mandibular base β angle 26 5 gonial angle 118.5 prognathism s n pg 84 inclination NSL/ML 23 5 Subject 1 23 year old

B Mean voltage during maximal bite AT 155 μV PT 137 μV MA 85 μV 118 μV
Mandibular base β-angle 23 0 gonial angle 130.5 prognathism s n pg 76

lated negatively to mandibular inclination as determined both by the mandibular line (NSL/ML $r = -0.35$ *) and the mandibular base line (NSL/MBL $r = -0.35$ *) Finally large activity in the masseter muscle was associated with small anterior height of both jaws (pr–pr $r = -0.45$ ** id–id $r = -0.34$ *) whereas the correlation to the total height of the anterior face was less pronounced (n–gn $r = -0.25$, $0.10 < p < 0.20$)

The mean voltage during maximal bite in *the internal pterygoid muscles* was large in subjects with both mandibular (s–n–pg $r = +0.36$ *) and maxillary prognathism (s–n–ss $r = +0.31$, $0.05 < p < 0.10$)

Depressor muscles

Chewing The activity in *the external pterygoid and the digastric muscles* during chewing began early in subjects with marked overbite (referred to the onset of activity in the right anterior temporal m, Table 37) Moreover the time dispersal between the onset of activity in the two depressor muscles and in the anterior temporal muscle was correlated negatively to the prognathism of both jaws The two muscles differed as regards the correlations to other morphological data early onset of the secondary activity in the external pterygoid muscles was associated with anterior inclination of the mandible (NSL/ML $r = +0.47$ ** NL/ML $r = +0.46$ **), strong activity in the digastric muscles followed the activity in the elevator muscles closely in subjects with proclination of the lower incisors and alveolar mandibular prognathism (IL_i/ML $r = +0.48$ ** CL/ML $r = +0.33$*) The time to onset of strong activity in *the mylohyoid muscles* during natural chewing (referred to the right anterior temporal m) tended to be correlated negatively to the overbite ($r = -0.29$ $0.05 < p < 0.10$) and positively to the inclination of the lower incisors (IL_i/ML $r = +0.37$ *)

The correlation between the overbite and the time to onset of activity in the external pterygoid and digastric muscles is exemplified in two subjects (Fig 78) Subject A (overbite 1 5 mm) had strong bursts of activity in the last part of the opening movement In subject B (overbite 5 mm) the activity was displayed continuously and in the digastric muscle it was less and more constant than in A

Swallowing The maximal mean voltage in *the external pterygoid muscles* during swallowing was correlated negatively to the inclination of the incisors and the arch length of the mandible the angle IL_i/ML and lower arch length decreasing with increasing activity (Table 38)

The occurrence in time of the strong activity in *the digastric muscles* during swallowing was positively correlated to the overjet negatively to

Table 37

Coefficients of correlation (r) between

trical activit\ the time to onset of activity in the external pterygoid and to the occurrence of strong activity in the digastric muscles during chewing

al morphology the cranial and mutual relationship of the jaws the inclination of the incisors and the alveolar prognathism in the lower jaw and the overbite

ubjects

acial morphology		Time to onset of (strong) activity during chewing (msec)(1)							
		Ext pterygoid muscles			Digastric muscles				
		Natural chewing			Natural chewing		Unilateral chewing		
Region	Ref points and lines (Fig. 71)	Onset of prim act	Onset of sec act	Cease of sec act	Time to 50 / MV_m	Time to MV_m	Time to 50 / $MV^{(2)}_m$	Time to Ipsilat	MV_m Contralat
xill									
rognath	s n ss	−0 38*	−0 23	−0 36	−0 15	−0 33*	−0 27	−0 49**	−0 35*
nclin	NSL/NL	−0 22	−0 02(3)	−0 15	−0 20	+0 03	−0 01	+0 002	+0 16
ndib									
rognath	s n pg	−0 28	−0 35*	−0 33	−0 23	−0 36*	−0.23	−0 35*	−0 29
nclin.	NSL/ML	+0 17	+0 47**	+0 24	+0 05	+0 07	−0 04	−0 01	+0 003
tual tionship he jaws									
agittal	ss n [illegible]	−0 09	+0 07	−0 01	+0 09	+0 05	−0 02	−0 09	−0 03
ertical	NL/ML	+0 28	+0 46 *	+0 34*	+0 14	+0 07(3)	−0 02	+0 01	−0 01
wer incisor lination	IL_i/ML	+0 07	−0 22	+0 04	+0 48**	+0 55 **	+0 45 *	+0 43**	+0 3?
wer eolar gnathism	CL/ML	−0 04	−0 01	−0 06	+0 33	+0 41*	+0 29	+0 25	+0 16
erbite	mm For def see Fig 73	−0 44**	−0 39*	−0 46	−0 53***	−0 53	−0 36*	−0 21	−0 25

Time zero was onset of activity in the right anterior temporal muscle

The distribution of the time data was positive skew ($\sqrt{b_1}$ = 0 99 1 / limit 0.92) since the other time data showed no departure from normality transformation was not applied

Departure from normality (0 001 < p < 0 01) of two-dimensional distribution

V mean voltage

angular measurements in degrees

n linear measurement in millimetres

0 01 < p < 0 05 r 0 001 < p < 0 01 r** p < 0 001

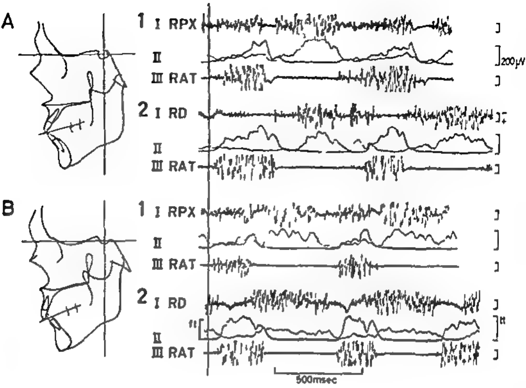


Fig 78

To illustrate the later onset of activity during chewing in the external pterygoid (primary act) *and digastric muscles in a subject with slight overbite* (A 1 5 mm) *than in a subject with marked overbite* (B 5 0 mm)

A The activity in the external pterygoid (primary act) and the digastric muscles appeared in the last half of the opening phase Subject 10 25¹/₂ year old

B The activity in the external pterygoid (primary act) and especially in the digastric muscles followed the activity in the reference muscle closely Subject 15 25 years old

Recordings obtained during natural chewing of apple *I* and thin mean voltage trace of *II* right external pterygoid (RPX 1) and right digastric (RD 2) muscles (test m) *III* and fat mean voltage trace of *II* right anterior temporal muscle (RAT ref m) Note the difference in calibration of thin (tt) and fat (ft) mean voltage traces in B 2 The vertical line indicates the onset of activity in the reference muscle RAT surface electrodes RPX and RD needle electrodes For definition of overbite see Fig 73

Table 38

Coefficients of correlation (r) between

electrical activity the maximal mean voltage in the external pterygoid muscles during swallowing

facial morphology incisor inclination alveolar prognathism arch length and spacing in the lower jaw

36 subjects

Facial morphology		Maximal mean voltage[1]				
		Reference RAT[2]			Reference RMY[2]	
Region	Ref lines (Fig 71)	Saliva µV	Saliva log µV	Apple µV	Saliva µV	Apple µV
Lower incisor inclination	IL_i/ML	−0 39*	−0 38*	−0 48**	−0 41*	−0 42**
Lower alveolar prognathism	CL/ML	−0 28	−0 22	−0 44**	−0 29	−0 28
Lower arch length	mm For def see Fig 73	−0 35*	−0 33	−0 42**	−0 27	−0 39*
Spacing in the lower jaw	mm For def see p 151	−0 18	−0 08	−0 26	−0 08	−0 31

[1] The four distributions of the maximal mean voltage (µV) were skew ($1\ 32 < \sqrt{b_1} < 1\ 65$ 1 / limit 0 92) Transformation was only applied to the maximal mean voltage during swallowing of saliva (Reference RAT) the transformed distribution (log µV) showed no departure from normality

[2] The analysis of correlation was carried out with the electromyographic data obtained both with the right anterior temporal (RAT) and with the right mylohyoid (RMY) as reference muscles

angular measurements in degrees

mm linear measurements in millimetres

r* $0\ 01 < p < 0\ 05$ r** $0\ 001 < p < 0\ 01$

Table 39

Coefficients of correlation (r) between

electrical activity the time of occurrence of the phase of strong activity in the digastric muscles du swallowing of saliva

facial morphology the cranial and mutual relationship of the jaws and the sagittal relationship of dental arches

Facial morphology			Time of occurrence of phase with strong activity (ref muscle RAT msec)[1]				
		Ref points and lines	Time to[2] 50 / MV_{ma}	Time to MV_m	Time to 50 / decline from MV_m		
Region		*(Fig 71)*	*36 subjects*	*36 subjects*	*36 subjects*	*32 subjects*[3]	*19 subjec*
Maxilla	prognath	s n ss	−0 02	+0 05[5]	+0 06	–	–
	inclin	NSL/NL	−0 26	−0 46**	−0 37*	−0 51*	−0 44 0 05<p<(
Mandible	prognath	s n pg	+0 01	+0 06	−0 004	–	–
	inclin	NSL/ML	+0 04	+0 02	+0 04	–	–
Mutual relation ship of the jaws	sagittal	ss n pg	−0 05	+0 03	+0 02	–	–
	vertical	NL/ML	+0 12	+0 16	+0 18	–	–
Sagittal occlusion of the molars	right side	mm	−0 10	−0 33*	−0 32	−0 19	−0 10
	left side	mm	−0 42**	−0 32	−0 33*	−0 43*	−0 31 0 2<p<0
Overjet		mm	+0 39*	+0 58***	+0 58***	+0 61***	+0 48*

[1] Time zero was onset of activity in the right anterior temporal muscle (RAT)

[2] The distribution of the time data was positive skew ($|\overline{b_1}| = 0\ 98$ 1 / limit 0 92) since the other t data showed no departure from normality transformation was not applied

[3] 4 subjects with low activity in the reference muscle (RAT) omitted

[4] 19 subjects with a maximal mean voltage in the reference muscle (RAT) during swallowing of 50 or more

[5] Departure from normality (0 001 < p < 0 01) of two-dimensional distribution

MV mean voltage

angular *measurements in degrees*

mm linear measurements in millimetres for def see Fig 73

r* 0 01 < p < 0 05 r** 0 001 < p < 0 01 r*** p < 0 001

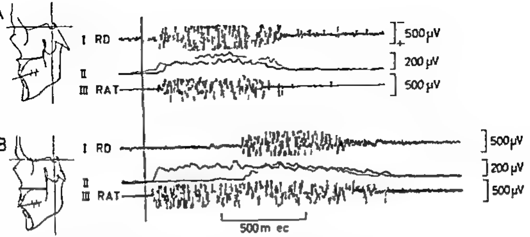


Fig 79

To illustrate the relation between the time to onset of strong activity in the digastric muscles during swallowing of saliva (time zero onset of activity in the right anterior temporal muscle) and the inclination of the maxilla and the sagittal relation of the incisors (overjet)

A. Simultaneous onset of activity in the digastric and the right anterior temporal muscles Maxillary inclination NSL/NL 6 0 Overjet 1 0 mm Subject 24 24 years old

B Delay between the onset of activity in the right anterior temporal and the digastric muscles Maxillary inclination NSL/NL 2 0 Overjet 5 0 mm Subject 30 24½ year old

Recordings obtained during swallowing of saliva *I* and thin mean voltage trace of *II* right digastric muscle (RD test m) *III* and fat mean voltage trace of *II* right anterior temporal muscle (RAT ref m) The vertical line indicates the onset of activity in the reference muscle RD needle electrode RAT surface electrode For definitions of morphological data see Fig 71 and 73

maxillary inclination and the sagittal relation of the molars the phase of strong activity in the digastric muscles occurred late during swallowing in subjects with anterior inclination of the maxilla, large maxillary overjet and Class II relationship of the molars (Table 39 Fig 79) These correlations were present only when the right anterior temporal served as reference muscle Aside from the sagittal relationship of the molars the same correlations were obtained (1) when omitting four subjects with low activity in the right anterior temporal muscles and (2) when considering 19 subjects with a maximal mean voltage in this muscle of 50 µV or more (cf Table 39 32 and 19 subjects) The total duration of activity during swallowing was not correlated to maxillary inclination and overjet hence these morphological traits were specifically related to the timing of the activity in the digastric muscles

Table 40

Coefficients of correlation (r) between

electrical activity the time to onset of strong activity in the mylohyoid muscle during swallowing of saliva

facial morphology the form of the cranial and mandibular bases the cranial and mutual relationship of the jaws the anterior height of the face and the jaws the overbite

36 subjects

Facial morphology			Time to onset of strong activity (time to 50 / MV_{ms} msec)[1]			
		Ref points and lines (Fig 71)	Reference muscle RMY[2]		Reference muscle RAT[3]	
Region			msec	log msec	msec	log msec
Cranial base		n-s-ar	+0.02	−0.02	+0.07	+0.11
Mandibular base		β-angle	+0.30	+0.31	+0.13	+0.07
		gonial angle	−0.34*	−0.33*	−0.15	−0.04
		pgn-cd mm	−0.01	+0.05	−0.02	+0.04
Maxilla	prognath	s n ss	+0.16	+0.09	+0.24	+0.14
	inclin	NSL/NL	−0.23	−0.21	−0.17	−0.10
Mandible	prognath	s n pg	+0.50**	+0.50**	+0.30	+0.30
		s-n-sm	+0.40	+0.34*	+0.30	+0.19
	inclin	NSL/ML	−0.43**	−0.41*	−0.30	−0.24
		NSL/MBL	−0.48**	−0.44**	−0.35*	−0.31
Mutual relation ship of the jaws	sagittal	ss n pg	−0.38*	−0.43**	−0.10	−0.13
	vertical	NL/ML	−0.39*	−0.36*	−0.24	−0.19
Ant face height		n gn mm	−0.38*[4]	−0.27	−0.34*	−0.23
Maxill zone		OL_s/NL	−0.33*	−0.37*	−0.18	−0.11
Ant maxill alv height		pr pr mm	−0.30	−0.29	−0.26	−0.18
Mandib zone		OL_i/ML	−0.28	−0.18	−0.34*	−0.29
Ant. mandib height		id id mm	−0.28	−0.23	−0.32	−0.38
Overbite		mm For def see Fig. 73	+0.07	+0.08	−0.16	−0.14

MV mean voltage

[1] The distributions of the time data (msec) were positive skew (reference RMY $\sqrt{b_1}$ = 1.79 reference RAT $\sqrt{b_1}$ = 1.52 1% limit 0.92) The transformed data (log msec) with RMY as reference showed no departure from normality those with RAT as reference showed a moderate negative skewness [illegible]01 < p < 0.05)

[2] Time zero onset of activity in the right mylohyoid muscle (RMY)

[3] Time zero onset of activity in the right anterior temporal muscle (RAT)

[4] Departure from normality (0.001 < p < 0.01) of two-dimensional distribution angular measurements in degrees β-angle and gonial angle see Fig. 72.

mm linear measurements in millimetre

r* 0.01 < p < 0.05 r** 0.001 < p < 0.01

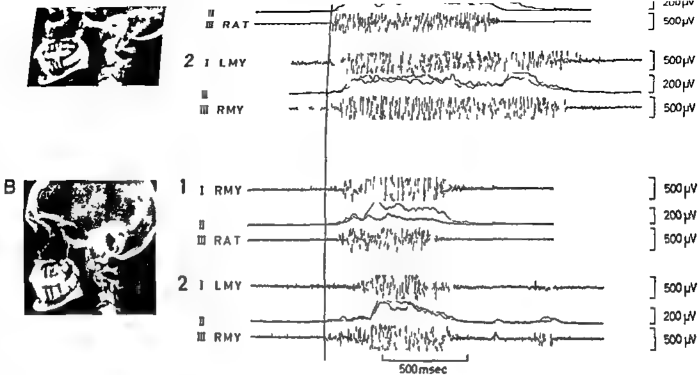


Fig 80

To illustrate the relation between the time of occurrence of strong activity in the mylohyoid muscle during swallowing of saliva (time zero onset of activity) and the prognathism and inclination of the mandible

A Early onset of strong activity in the mylohyoid muscles Mandibular prognathism s n pg 80 5 mandibular inclination NSL/ML 30 0 Subject 15 26 years old

B Delayed onset of strong activity in the mylohyoid muscles Mandibular prognathism s n pg 85 0 mandibular inclination NSL/ML 18 5 Subject 20 23½ year old

Recordings obtained during swallowing of saliva *I* and thin mean voltage trace of *II* right (1 RMY) and left (2 LMY) mylohyoid muscles (test m) *III* and fat mean voltage trace of *II* right anterior temporal (1 RAT) and right mylohyoid (2 RMY) muscles (ref m) The vertical line indicates the onset of activity in the reference muscles RAT surface electrodes RMY and LMY needle electrodes For definitions of morphological data see Fig 71

Table 41

Coefficients of correlation (r) between

electrical activity the average level of the mean voltage in the external pterygoid muscles with the mandible at rest and in the digastric muscles with the mandible at rest and in the occlusal position

facial morphology the inclination of the incisors and the alveolar prognathism in the lower jaw the width perimeter and spacing of the upper and lower arches

36 subjects

Facial morphology			Ext pterygoid *Mean volt* age with mandible at rest	*Digastric* Mean voltage with man dible at rest and in the occlusal position[1]	
Region		Ref lines (Fig 71)	µV	µV	log µV
Lower incisor inclination		IL_i/ML	−0 39*	−0 32	−0 39*
Lower alveolar prognathism		CL/ML	−0 23	−0 45**	−0 44**
Upper arch	width	mm For def see Fig 73	−0 47**	−0 16	−0 09
	perimeter	mm For def	−0 30	−0 11	+0 02
	spacing	mm see p 151	−0 12	+0 05	+0 16
Lower arch	width	mm For def see Fig. 73	−0 38*	−0 14	−0 08
	perimeter	mm For def	−0 45**	−0 39*	−0 29
	spacing	mm see p 151	−0 27	−0 35*	−0 27

[1] The variance analysis showed a random variation between the mean voltage with man dible at rest and in the occlusal position to calculate the coefficients of correlation data from the two positions were averaged in each subject The distribution of the mean voltage (µV) was positive skew ($|\sqrt{b_1}$ = 2 04 1 / limit 0 92) and platykurtic (a = 0 69 1 limit 0 72) after transformation (log µV) the distribution showed no departure from normality

angular measurements in degrees

mm linear measurements in millimetres

* 0 01 < p < 0 05 ** 0 001 < p < 0 01

In *the mylohyoid muscle* (Table 40 Fig 80) the time from onset of activi ty to the onset of strong action was positively correlated to mandibular prog nathism (s–n–pg. r = +0 50 **) negatively to mandibular inclination (NSL/ML r = −0 43 * NSL/MBL r = −0 48 **) subjects with strong activity in the mylohyoid muscle from the very beginning of swallowing were char acterized by retrognathism and posterior inclination of the mandible and had a flattened mandibular base (β-angle r = +0 30 0 05 < p < 0 10 gonial angle r = −0 34) These correlations were less pronounced with

the right anterior temporal as reference muscle The time dispersal between the onset of activity and the onset of strong activity in the mylohyoid muscle was the only time parameter of this muscle correlated to morphology

The maximal mean voltage in the right mylohyoid muscle (as reference muscle) was negatively correlated to the overbite ($r = -0.38$ *) subjects with strong activity during swallowing tended to have an open bite This correlation was not found in the experiments in which the right and left mylohyoid muscles were test muscles ($r = -0.01$) the association with the overbite was only revealed when the maximal mean voltage during swallowing was determined as the average from several experiments This could be expected in view of the large change in mean voltage which may occur because of a small change in the position of the electrode (cf Fig 19 and Fig 50 B)

Postural activity (Table 41) The activity in *the external pterygoid muscle* with the mandible at rest was correlated negatively to the inclination of the lower incisors ($r = -0.39$ *) to the width of the dental arches (upper $r = -0.47$ ** lower $r = -0.38$ *) and to the perimeter of the lower arch ($r = -0.45$ **), the postural activity in *the digastric muscles* was correlated negatively to the inclination of the incisors the alveolar prognathism and the spacing in the mandible (IL$_i$/ML $r = -0.32$ $0.05 < p < 0.10$ CL/ML $r = -0.45$ ** spacing $r = -0.35$ *) Hence large postural activity in these muscles was observed in subjects with retroclination of the incisors small alveolar prognathism and crowding in the mandible

Subjects with strong activity in the external pterygoid muscles during swallowing had retroclination of the lower incisors (cf Table 38) The similar association with the postural activity may therefore indicate a constant high level of activity in these subjects

Orbicularis oris muscles

Chewing Strong activity in the lower lip during the opening movement (primary activity) was associated with prognathism of both jaws in the case of the lower jaw however only with the prognathism corresponding to the apical base (Table 42) The positive correlation between the primary activity in the lower lip and prognathism existed both during natural and unilateral chewing

The degree of activity in the muscles of the lower lip during chewing was related to lip posture in two subjects (Fig 81) the first subject (A) had a distinct mentolabial sulcus and the innervation of the lower lip during chew

Table 43

Coefficients of correlation (r) between

electrical activity the maximal mean voltage in the upper lip during the closing movement during chewing (secondary activity)

facial morphology the inclination of the upper incisors and the overbite

36 subjects

Facial morphology		Maximal mean voltage (µV)		
		Natural chewing[1]		Unilateral chewing
Region	Ref lines (Fig 71)	µV	log µV	µV
Upper incisor inclination	IL_s/NL	-0.45^{**}	-0.43^{**}	-0.39^{*}
Overbite	mm	$+0.34^{*}$	$+0.39^{*}$	$+0.34^{*}$

(1) The distribution of the maximal mean voltage (µV) was positive skew ($\sqrt{b_1} = 1.57$ 1% limit 0.92) the transformed data (log µV) showed no departure from normality

° angular measurement in degrees

mm linear measurement in millimetres for def see Fig 73

r^{*} $0.01 < p < 0.05$ r^{**} $0.001 < p < 0.01$

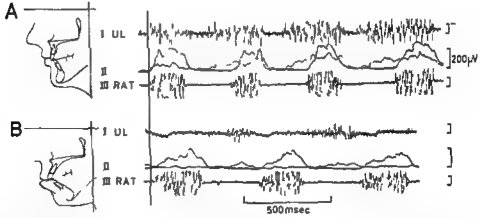


Fig 82

To illustrate the larger activity in the upper lip during natural chewing of bread in a subject with retroclination of the upper incisors (A) than in a subject with proclination of the incisors (B)

A Strong activity in the upper lip simultaneously with the action of the right anterior temporal muscle (secondary act) Inclination of upper incisors IL /NL 97.0 Subject 22 25 years old

B Weak activity in the upper lip during the opening movement (primary act) and almost no activity during the closing movement (secondary act) Inclination of upper incisors IL /NL 119.5 Subject 23 22½ year old

I and thin mean voltage trace of *II* upper lip muscles (UL test m) *III* and fat mean voltage trace of *II* right anterior temporal muscle (RAT ref m) The vertical line indicates the onset of activity in the reference muscle Surface electrodes For definition of IL NL see Fig 71

Table 44

Coefficients of correlation (r) between
electrical activity the maximal mean voltage in the upper and lower lip during swallowing of saliva and apple
facial morphology the cranial and mutual relationship of the jaws the inclination of the incisors and the alveolar prognathism in the lower jaw overjet overbite and spacing

36 subjects

Facial morphology			Maximal mean voltage during swallowing[1]						
Region		Ref points and lines (Fig 71)	Upper lip Saliva µV	Upper lip Saliva log µV	Upper lip Apple µV	Upper lip Apple log µV	Lower lip Saliva µV	Lower lip Saliva log µV	Lower lip Apple µV
Maxilla	prognath	s-n-ss	+0.04	−0.01	−0.10	−0.04	−0.04	−0.02	−0.07
	inclin.	NSL/NL	−0.20	−0.29	−0.14	−0.02	−0.19	−0.19	−0.09
Mandible	prognath	s-n-pg	−0.21	−0.27	+0.17	+0.24[2]	−0.17	−0.15	−0.14
		s-n-sm	−0.21	−0.17	−0.15	−0.19	−0.19	−0.14	−0.04
	inclin	NSL/ML	−0.04	−0.17	−0.12	−0.21	−0.12	−0.18	−0.04
Mutual relationship of the jaws	sagittal	ss-n-pg	−0.22	−0.28	−0.34	−0.34*	−0.26	−0.18	−0.19
	vertical	NL/ML	*−0.01*	*−0.09*	*−0.28*	*−0.21*	*−0.03*	*−0.14*	*−0.14*
Lower incisor inclination		ILi/ML	−0.36	−0.28	−0.34*	−0.32	−0.26	−0.09	−0.50
Lower alveolar prognathism		CL/ML	−0.19	−0.24	−0.21	−0.25	−0.11	−0.01	−0.42 **
Overjet		mm	−0.24	−0.21	−0.05	−0.12	−0.21	−0.22	−0.09
Overbite		mm	−0.08	−0.12	−0.11	−0.08	−0.10	−0.12	−0.23
Spacing	maxilla	mm	−0.15	−0.11	−0.21	−0.20	−0.13	−0.06	−0.19
	mandible	mm	−0.07	−0.06	−0.37	−0.28	−0.21[2]	−0.01	−0.32

[1] The maximal mean voltage (µV) in the upper lip during swallowing of saliva and apple and in the lower lip during swallowing of saliva showed positive skew distributions ($1.59 < \sqrt{b_1} < 1.75$ 1 limit 0.92) after transformation skewness was absent but the activity in the lower lip showed a moderate platy kurtosis ($0.01 < p < 0.05$)

[2] Departure from normality ($0.001 < p < 0.01$) of two-dimensional distribution.

° angular measurements in degrees

mm linear measurements in millimetres for def see Fig. 73 and p 151

* $0.01 < p < 0.05$ r** $0.001 < p < 0.01$

Swallowing Strong activity in the lips during swallowing was associated with retroclination of the lower incisors strong activity during swallowing of apple was associated with crowding in the lower arch (Table 44) The correlations to the activity during swallowing differed from those to the activity during chewing and with the mandible at rest (Table 42) as regards the sagittal relation of the jaws and mandibular alveolar prognathism tendencies were opposite (activity during chewing and at rest, ss–n–pg $+0.19 < r < +0.31$ CL/ML $+0.11 < r < +0.24$ activity during swallowing, ss–n–pg $-0.19 < r < -0.34$* CL/ML $-0.11 < r < -0.42$**)

Table 43

Coefficients of correlation (r) between

electrical activity the maximal mean voltage in the upper lip during the closing movement during chewing (secondary activity)

facial morphology the inclination of the upper incisors and the overbite

36 subjects

Facial morphology		Maximal mean voltage (μV)		
		Natural chewing[1]		Unilateral chewing
Region	Ref lines (Fig 71)	μV	log μV	μV
Upper incisor inclination	IL_s/NL	−0 45**	−0 43**	−0 39*
Overbite	mm	+0 34*	+0 39*	+0 34*

(1) The distribution of the maximal mean voltage (μV) was positive skew ($\sqrt{b_1}$ = 1 57 1 / limit 0 92) the transformed data (log μV) showed no departure from normality angular measurement in degrees

mm linear measurement in millimetres for def see Fig 73

r* 0 01 < p < 0 05 r** 0 001 < p < 0 01

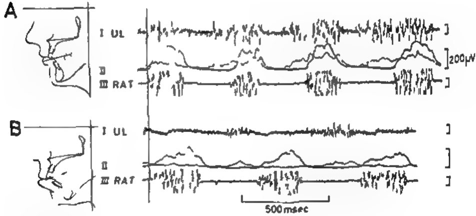


Fig 82

To illustrate the larger activity in the upper lip during natural chewing of bread in a subject with retroclination of the upper incisors (A) *than in a subject with proclination of the incisors* (B)

A Strong activity in the upper lip simultaneously with the action of the right anterior temporal muscle (secondary act) Inclination of upper incisors IL_s/NL 97 0 Subject 22 25 years old

B Weak activity in the upper lip during the opening movement (primary act) and almost no activity during the closing movement (secondary act) Inclination of upper incisors IL /NL 119 5 Subject [redacted] 22½ year old

I and thin mean voltage trace of *II* upper lip muscles (UL test m) *III* and fat mean voltage trace of *II* right anterior temporal muscle (RAT ref m) The vertical line indicates the onset of activity in the reference muscle Surface electrodes For definition of IL NL see Fig. 71

tongue begins to bring the saliva backwards (as indicated by the activity in the mylohyoid muscles Fig 61) In the following an attempt is made to clarify the relation between the activity in the lips during swallowing and lip posture by considering the coordination of the orbicularis oris and mylohyoid muscles in the initial phase of swallowing (Fig 83 subjects A B, C and D)

In subject *A* action of the lips was almost absent the anterior seal most likely being established passively by jaw closure In subject *B* a moderate

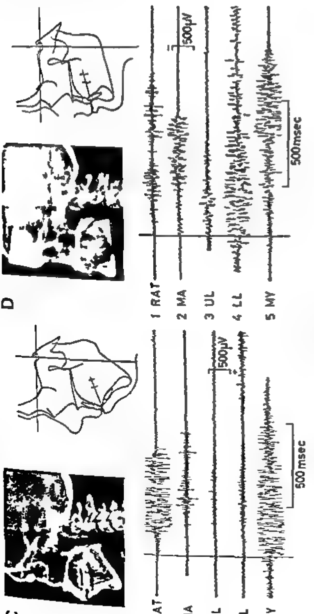


Fig 83 continued

C Insufficient lips More than average activity in the temporal (1) and average activity in the masseter (2) muscles Weak activity in the lips (3 4) simultaneously with the elevator muscles Early onset of strong activity in the mylohyoid muscles (5) Subject 14 24 years old

D Lips closed Average activity in the temporal (1) and more than average activity in the masseter (2) muscles *Marked activity in the upper lip (3) strong activity in the lower lip (4) and a prolonged initial* phase with low activity in the mylohyoid muscles (5) Subject 2 23½ year old

The vertical lines indicate the onset of activity in the right anterior temporal muscle (ref m) RAT right anterior temporal MA masseter UL upper lip LL lower lip MY mylohyoid surface electrodes 1–4 needle electrode 5

degree of insufficiency was indicated by the small contact area between the upper and lower lip with the mandible in the intercuspal position The electromyograms showed strong action of the lips before activation of the mylohyoid muscles Subject C presented the most posterior inclination of the mandible among the 36 subjects examined (NSL/ML 47 0°, NL/ML 36 5°) This trait appeared in the profile tracings which in addition indicated that the lips were insufficient to obtain contact even with the mandible in the intercuspal position The electromyograms from this subject differed from those of subject *B* in two respects (1) the activity in the orbicularis oris muscles was low and occurred simultaneously with that of the elevator muscles and (2) the strong activity in the mylohyoid preceded the elevator muscles The early and abrupt onset of activity in the mylohyoid muscles indicated strong action of the tongue in the very first part of swallowing possibly the tongue established the anterior seal and compensated for the insufficient lips

Hence the activity in the orbicularis oris muscles during swallowing was not determined solely by the relation between the anterior aspects of the jaws and the size of the lips The recordings from subject *D* provided additional evidence in spite of closed lips this subject swallowed with strong activity in the lower lip muscles whereas the initial phase of low innervation in the mylohyoid muscles was prolonged

DISCUSSION

Previous studies of the relation between the action of the muscles of mastication and facial morphology concerned comparisons between the function in subjects with normal and abnormal dental occlusion dealing mainly with (1) the influence of the elevator muscles on sagittal and vertical occlusion and (2) the significance of the lips and the tongue for the labio-lingual development of the dental arches In the study presented in this report the correlation was analysed between oral function (chewing swallowing posture and full effort) and sagittal and vertical facial dimensions The correlations are discussed separately as they concern elevator depressor and lip muscles and as related to the principles underlying the development in shape and size of the face

Elevator muscles

Chewing The relation between the degree of activity in the temporal and masseter muscles during chewing and the dental occlusion was studied by

Ahlgren (1966) without evidence of a difference between normal and mal occlusion. In my experiments the activity in the anterior and posterior parts of the temporal muscle was recorded separately and strong activity in the posterior parts was found in subjects with low anterior height of the upper alveolar process retroclination of the upper incisors and marked overbite (Table 31)

According to *Witt* (1961 1963) and *Ahlgren* (1966) a small gonial angle was associated with strong activity in the masseter muscles during chewing *Witt* (1961 1963) used the average spike voltage in electromyograms obtained during natural chewing of bread as a measure of the degree of activity (one minute of recording) *Ahlgren* (1966) used integration of the electrical activity over a period of 20 sec during unilateral chewing of gum In my recordings the maximal mean voltage in the masseter muscles during natural chewing showed only a tendency of a correlation to the gonial angle (ML/RL apple $r = -0\ 13$ $0\ 40 < p < 0\ 60$ bread $r = -0\ 28$ $0\ 05 < p < 0\ 10$) Since neither *Witt* (1961 1963) nor *Ahlgren* (1966) tested their findings statistically further investigations are required to decide whether a relation exists between the action of the masseter mu cles during chewing and the shape of the mandible

Perry and *Harris* (1954) and *Perry* (1955 1961) found that the masseter muscles were activated earlier during unilateral chewing in subjects with Class II Div 1 occlusion than in subjects with normal occlusion. In the subjects examined in this study the occurrence in time of the activity in the masseter muscles during chewing showed no correlation to the relation of the jaws and the dental arches in the sagittal plane On the other hand early activation of the masseter muscles in subjects with a flattened cranial base might be in keeping with an association with a severe Class II relationship (cf Table 32 Fig 75)

Swallowing In previous studies of children (*Baril* and *Moyers* 1960) and adults (*Findlay* and *Kilpatrick* 1960) there was no evidence of a relation between the electrical activity in the elevator muscles during swallowing and morphological data The present material showed that strong activity in the masseter muscles was associated with total prognathism strong activity during swallowing in the temporal muscles was found in subjects with a curved cranial base

Postural activity *Moyers* (1949) believed that strong postural activity in the posterior temporal muscles was associated with mandibular retrognathism an assumption which was not statistically verified In fact there was a tendency of a positive correlation between the mean voltage in the posterior

temporal muscle with the mandible at rest and the cranial relationship of the mandible (see Table 35) moreover subjects with large postural activity in the posterior temporal muscles were characterized by maxillary prognathism

Full effort The force during maximal bite estimated by the mean voltage in the anterior temporal and in the masseter muscles was correlated positively to mandibular prognathism and negatively to mandibular inclination Malocclusion may be associated with both prognathism and anterior inclination (large activity) and retrognathism and posterior inclination of the mandible (small activity) It is therefore unlikely to expect a difference between the activity during maximal bite in subjects with normal occlusion and malocclusion unless the type of malocclusion is specified This explains why *Friel* (1924 1926) and *Lancet* (1927) found no difference between the biting force on the molars of subjects with normal and abnormal dental occlusion *Johnson* and *Hatfield* (1917) found a smaller biting force in malocclusion than in normal occlusion however their interpretation is complicated by the subjects with very poor dentition in their group with malocclusion

The coordination of the elevator muscles during biting on the incisors has been used to indicate an anterior position of the mandible in subjects with malocclusion (reduced activity in the anterior temporal m predominance of the masseter m see p 144, incisive bite. *Grossmann* and *Greenfield* 1956 *Timms* 1960 *Grossmann* et al 1961 *Timms* and *Greenfield* 1961) This may be true when cusp interference displaces the mandible forward during biting When mandibular prognathism was a morphological trait it was connected with large activity during maximal bite in both the anterior temporal and in the masseter muscles The reduced activity in the anterior temporal muscle during biting with the mandible in protrusion can explain the larger activity in the masseter muscles at a given biting pressure in Class II subjects as compared with subjects with normal occlusion (*Garrett* et al 1964, see p 14) Class II subjects must protrude their mandible most to be able to bite on the pressure transducer

Depressor muscles

Previous mechanical (*Friel* 1926) and electromyographic (*Schlossberg* 1954 *Blenker* 1955 *Krazer* 1960) investigations of the depressor muscles showed no conclusive correlations to facial morphology I found that

(a) early activation of the external pterygoid and digastric muscles during chewing was associated with a marked overbite (Table 37)

(b) large activity in the external pterygoid muscles during swallowing was connected with retroclination of the lower incisors and a short lower arch (Table 38)
(c) strong activity in the digastric muscles during swallowing occurred late in subjects with maxillary overjet (Table 39)
(d) strong activity in the mylohyoid muscles during swallowing occurred early in subjects with a posteriorly inclined mandible and a flattened mandibular base (Table 40) and
(e) subjects with large postural activity in the external pterygoid and digastric muscles had retroclination of the lower incisor and a tendency of crowding in the lower arch (Table 41)

Orbicularis oris muscles

The relation between the function of the lips and the dental occlusion has been studied extensively (*Friel* 1924 1926 *Lancet* 1927 *Winders* 1958 1962 *Baril* and *Moyers* 1960 *Werner* 1964 *Witt* 1964) without evidence of differences between abnormal and normal occlusion My findings agree with the exception that the secondary activity in the upper lip during chewing was related to the degree of overbite (Table 43 Fig 82)

Recording with surface electrodes does not allow the action of the individual muscles of the lips to be distinguished (see p 21) the correlations between their activity during chewing and morphology is assumed to indicate the following differentiation

1 An anterior seal during the opening movement of chewing is established by the orbicularis oris proper by fibres originating from the maxilla and contributing to the lower lip and by fibres originating from the mandible and ending in the upper lip The activation of these muscles is assumed to depend on the anterior sagittal development (s–n–ss s–n–sm)

2 Placement of the bolus between the teeth in the closing movement of chewing requires a pressure directed lingually obtained by the innervation of the buccinator muscles This activity appears (mechanically and electromyographically) in the incisor area and may explain the relationship between the secondary activity in the upper lip and the inclination of the upper incisors and the overbite

It has been shown that the activity in the lips during swallowing increased with the degree of insufficiency of the lips (Fig 83 A and B) However when the insufficiency was severe (Fig 83 C) the lips were almost passive and the early activation of the mylohyoid muscles is assumed to represent a compensatory action of the tongue This pattern agrees with (1) the ratio between the pressure of the lips and of the tongue observed in subjects with

relapse after treatment of anterior open bite (*Kydd* et al 1963) and (2) the larger separation of the lips during swallowing in subjects with tongue thrust (*Cleall* 1965)

Muscle disease and animal experiments

Progressive muscular dystrophy is the only muscle disease in which a specific type of malocclusion—an anterior open bite—has been described (*Brown* and *Losch* 1939 *White* and *Sackler* 1954) *In animal experiments* bilateral excision of the masseter muscles resulted in overeruption of the molars and anterior open bite (*Horowitz* and *Shapiro* 1955 rats *Schumacher* 1964 rabbits) However the description of the morphological data in patients with muscular dystrophy (*Brown* and *Losch* 1939 *White* and *Sackler* 1954) and in animal experiments (*Horowitz* and *Shapiro* 1955 *Schumacher* 1964) are incomplete and do not allow a comparison with the correlations found between the activity in the elevator muscles and the mutual relation of the jaws in the vertical plane A study of the development of the facial skeleton and the elevator muscles in children with muscular dystrophy could possibly clarify whether or not a connection exists between the anterior open bite (*Brown* and *Losch* 1939, *White* and *Sackler* 1954) and the masting of the elevator muscles

Activity in the muscles of mastication and formative development *)

The formative development of the face is closely associated with the development of the cranial and mandibular bases A curved *cranial base* is associated with a forward–downward position and an anterior inclination of both jaws (total prognathism) a flattened cranial base is associated with posterior inclination of the upper and lower jaw (total retrognathism) The maximal mean voltage in the masseter muscle during swallowing was correlated to the different traits of facial prognathism the larger the activity the more prognathous was the facial skeleton the activity in both parts of the temporal muscles during swallowing was correlated only to the curvature of the cranial base large activity being associated with a curved cranial base

The mandibular base may be arch shaped with a high ramus, a large β-angle and a small gonial angle (cf Fig 72) and the mandible is then anteriorly inclined and prognathous subjects with a flattened mandibular base have a posteriorly inclined and retrognathous mandible The shape of

*) In the following discussion the principles of facial development were taken from *Bjork* (1958 b 1960 and 1963 a)

the mandibular base the prognathism and the inclination of the mandible were correlated to the following two electromyographic magnitudes (1) the activity (average level of the mean voltage) in the anterior temporal and in the masseter muscles during maximal bite in the intercuspal position (Table 36) (2) the difference in time between the onset of activity and the onset of strong activity in the mylohyoid muscles during swallowing (Table 40)

The activity in the temporal and masseter muscles during maximal bite represented in most subjects the upper limit of the activity during chewing and swallowing (cf Fig 68) Therefore the activity during maximal bite could serve as a measure of the development of these muscles A great strength of the elevator muscles (especially the masseter m) might be assumed to be associated with the development of a large angulus of the mandible causing the mandible to appear anteriorly inclined However this assumption is unlikely since (1) the shape of the mandibular base primarily is determined by the direction and the amount of condylar growth and (2) studies by means of metallic implants (*Bjork* 1963 b) have shown that resorption at the lower border of the ramus is especially large in subjects with vertical condylar growth i e with anterior inclination of the mandible Orthodontic appliances might be assumed to exert influence (stimulating and retarding) on the centres of growth i e on the development of the cranial and mandibular bases (*Bjork* 1963 a) A similar influence of the elevator muscles could possibly explain the correlation between their activity and the form of the cranial and mandibular bases

The early onset of strong activity in the mylohyoid muscles during swallowing in the case of insufficient lips (Fig 83 C) is interpreted in terms of an intense (compensatory) action of the tongue in the beginning of swallowing Hence the correlation between the prognathism inclination and form of the mandible and the timing of the activity in the mylohyoid muscles during swallowing may in fact be ascribed to a variation in the function of the tongue To substantiate this assumption would require a closer analysis of the coordination between the mylohyoid and the tongue muscles

The cranial relationship of the maxilla was related to the coordination in time of the strong phase of the activity in the digastric muscles during swallowing subjects with a large time dispersal between the onset of swallowing and the strong action of the digastric muscles had an anterior inclination of the maxilla and a maxillary overjet The time of strong activity in the digastric muscles corresponded to the pharyngeal phase of swallowing (cf Fig 61) Since maxillary inclination and overjet showed no correlation to the total duration of swallowing they might be connected with the mutual time relation of the oral and pharyngeal phases

As to *the dentoalveolar relationships* an increased overbite was found in

subjects with (a) strong activity in the posterior temporal muscles during maximal bite and chewing, and (b) early activation of the depressor muscles during chewing. These correlations could depend on the degree of over bite a large overbite necessitates (a) a retrusion of the mandible during closing and (b) precise movements during opening to avoid that the incisors interfere with the movements during chewing.

The retroclination of the lower incisors in subjects with strong activity in the external pterygoid muscles during swallowing might be caused by a forward thrust of the mandible This assumption cannot explain why retroclination of the incisors and small alveolar prognathism also was seen in subjects with strong postural activity in the external pterygoid and the digastric muscles

Facial morphology and the function of the lips Findings as to the relation between the morphological data and the activity in the orbicularis oris were not uniform In some instances the action of the lips was related to a discrepancy between the form and size of the facial skeleton and the size of the lips the primary activity in the lower lip during chewing was large in subjects with prognathism of both jaws (Table 42) That the activity in the lower lip increased with facial prognathism without being correlated to the alveolar prognathism and the inclination of the incisors may indicate a compensatory effect of the lips on the dental and alveolar arches (*Bjork* 1953) In other instances the variation of the dento-alveolar relationships most likely depended on the activity in the lips, this concerned the retroclination of the lower incisors in subjects with strong activity during swallowing Finally the activity in the lips was related to the coordination in time of the mylohyoid muscles insufficient lips were activated slightly when the mylohyoid muscles were activated early during swallowing (Fig. 82 C), whereas strong action of the lips was associated with a long initial phase with low activity in the mylohyoid muscles (Fig. 83, D) Hence irrespective of the degree of activity the action of the lips must be considered in connection with the morphological and functional conditions before its importance for the etiology of malocclusion can be estimated

The study presented in this report has demonstrated a number of correlations between the activity in the muscles of mastication and the variation in the form and the size of the cranial base the jaws and the dental and alveolar arches That these correlations were unnoticed in previous investigations was due to the unprecise classification of the morphological and functional data. The disadvantages of Angle s classification in the analysis of the relation between the shape of the jaws and dental occlusion were pointed out by *Bjork* (1947) these disadvantages were also present when the dental occlusion was related to muscle activity

Classification of functional findings mainly concerned the patterns of muscle activity during swallowing This study has shown that the degree of activity in the elevator lip and suprahyoid muscles varies independently during swallowing Therefore the classification into a normal and abnormal type is insufficient to describe the activity in the muscles during swallowing

It emerged from the present study that comparing each morphological trait separately with muscle activity in terms of duration amplitude and timing was a profitable way of analysing the relation between form and function

SUMMARY

The aim of the present study was (1) to evaluate the effect of the physical variables (impedance and position of the electrodes, input impedance of the amplifier frequency response of the recording system) on electromyographic recordings from the muscles of mastication, (2) to describe quantitatively the degree and the time course of the electrical activity in the elevator depressor and lip muscles in young adult males during chewing and swallowing at rest and during full effort, (3) to determine the time of make and break of tooth contact during chewing and swallowing and (4) to analyse the correlation between the electrical activity in the muscles and the morphology of the cranial base, the jaws and the dental and alveolar arches

METHOD

Part of the methodological investigations were made by recording with an eight channel electroencephalograph with inkwriters (KAISER type no E 1000 input impedance 2MΩ in parallel with 2000 pF frequency response 3 db down at 1 5 and 115 cycle/sec) The inkwriter recordings were measured graphically (Møller 1958) Additional methodological experiments and all investigations of muscle activity during natural function were carried out by means of a three channel electromyograph with photographic recording from cathode ray oscilloscopes (DISA type no 13A50 input impedance 100 MΩ in parallel with 60 pF frequency response 3 db down at 2 and 10 000 cycle/sec the lower limiting frequency was increased to 20 cycle/sec to reduce artefacts from movements of the electrodes) One amplifier of the electromyograph was replaced by an apparatus for determining the numerical mean voltage of the electrical activity (DISA type no 13B05) By means of this set up two electromyograms and their mean voltages were recorded simultaneously

In the *static experiments* (methodological studies and the activity at rest and during full effort) the degree of electrical activity was determined by the mean voltage in recordings obtained with a speed of 5 cm per sec

The time course of the electrical activity in the *dynamic experiments* (chewing and swallowing) was characterized by (1) the time of onset and cessation of the activity determined from the directly recorded electromyograms and (2) the phase of contraction with the highest degree of activity measured from the time when the mean voltage had reached 50 per cent of its maximum to the time when it had declined to 50 per cent In addition this phase was described by the maximal mean voltage and by the time of occurrence of this maximum In the dynamic experiments the recording speed was 20 cm per sec To determine the onset and cessation of the activity with sufficient accuracy the electromyograms were recorded with a high and constant amplification (1 mm = 20 μV)

METHODOLOGICAL INVESTIGATIONS

The methodological investigations were performed on the temporal masseter and brachial biceps muscles and comprised 33 subjects 5 females and 28 males 18–49 years of age

Impedance of electrodes The impedance of surface electrodes depended on individual differences between the electrodes on their location and on the preceding treatment of the skin electrodes placed on the temporal muscle had impedances similar to or larger than those of concentric needle electrodes (100–200 kΩ at 50 cycle/sec) the impedance of electrodes on the masseter muscle corresponded to the impedance between two earth electrodes (1 kΩ $< |Z| <$ 2 kΩ at 50 500 and 5000 cycle/sec) the impedance between two surface electrodes was reduced up to 70 per cent by repeated rubbing of the skin with ether

Difference amplification Difference amplifiers transmit a potential difference between the two leads of the recording electrode core and cannula of the concentric needle electrode single leads of the bipolar or unipolar surface electrode a common voltage of the two leads (for instance caused by activity in adjacent muscles) is more or less rejected With surface electrodes the difference amplification was high as compared to the amplification of a common voltage both with a low and a high input impedance (1 8 MΩ 60 pF 50 1 at 100 cycle/sec 100/1 above 500 cycle/sec 100 MΩ 60 pF $>$ 200/1 between 5 and 500 cycle/sec) With concentric needle electrodes and low input impedance (1 8 MΩ 60 pF) the ratio of difference amplification to the amplification of a common voltage was less than 20/1 between 5 and 50 cycle/sec Thus a localized recording with concentric needle electrodes is

only obtained when they are used in connection with an amplifier with a high input impedance

Input impedance and frequency response of the amplifier When an ink writer (electroencephalograph) was used for recording the electrical activity in the brachial biceps muscle (loads 1–10 kg) the mean voltage was 25 per cent (surface electrodes) and 60 per cent (needle electrodes) lower than when recording was with cathode ray oscilloscope on photographic paper (electromyograph) Voltage division between the electrode and electroencephalograph due to the low input impedance of the latter can account for 10–15 per cent of the reduction of the mean voltage The additional decrease was due to the low upper limiting frequency of the electroencephalograph Hence the mean voltage of the electromyogram depends both on the input impedance of the amplifier (voltage division) and its upper limiting frequency (amplification of spike potentials)

Bipolar and unipolar surface electrodes When one of the electrodes was placed at some distance from the muscle under study (unipolar placement) activity was led off from adjacent muscles With bipolar recording and a distance between the leads which is small as compared to the size of the muscle conducted activity from other muscles is nearly identical on the two leads and therefore rejected

Bipolar surface recording The distance between the electrodes, their position on the muscle and their size must be kept constant since the mean voltage varies with these magnitudes

Distribution of the electrical activity during isometric contraction Recordings with a multilead electrode from the brachial biceps muscle during a constant load showed that the degree of activity could differ by a factor of two between closely situated (2 mm) sites of recording This observation must be taken into account before a difference in mean voltage is interpreted as representing a difference in muscle function

ELECTRICAL ACTIVITY IN THE MUSCLES OF MASTICATION DURING NATURAL FUNCTION

The study of the natural function of the muscles of mastication and its correlation to the morphology of the chewing apparatus was performed on 36 male dental students 20–30 years of age with all teeth preserved (except

the third molars), without history of orthodontic treatment and without signs or symptoms of neuromuscular disorders or involvement of the temporomandibular joints The investigation included the following muscles (1) the anterior and (2) posterior temporal (3) the masseter (4) the internal and (5) external pterygoid (6) the digastric (7) the mylohyoid muscles and the muscles of the upper (8) and lower lip (9) Recordings from the muscles 1–7 were bilateral the activity in 1–3 was led off by bipolar surface electrodes (3 × 10 mm) placed with a distance of 10 mm along the direction of the fibres and with their long axis transversely to the direction of the fibres the electrodes on the muscles of the lips were smaller (3 × 5 mm) but placed as on 1–3 From the muscles 4–7 the activity was led off by concentric needle electrodes During chewing and swallowing the time of make and break of contact was determined on the incisors (36 subjects) and molars (10 subjects) With a two-channel recording system (2 electromyograms and their mean voltages) the activity in the different muscles was compared by keeping one muscle (reference muscle) in constant connection with one of the recording channels The activity in the other muscles (test muscles) and the signals of tooth contact were recorded successively on the other channel Time zero of all measurements of time was the onset of activity in the reference muscle (right anterior temporal muscle)

Mastication

The study of chewing comprised natural chewing of apple and bread and unilateral chewing of chewing gum During each chewing stroke the electrical activity in *the right anterior temporal muscle* (reference m) lasted about 300 msec and strong activity was present for less than 200 msec Strong activity occurred earlier during natural than during unilateral chewing the further time course of the activity (total duration time of maximal activity and the cessation of the period of strong activity) was the same during chewing of apple and bread as during ipsilateral and contralateral chewing of gum The total duration of the chewing cycle (580–680 msec) was longer during chewing of bread than during chewing of apple it had the longest duration during unilateral chewing These differences in the duration of the chewing cycle concerned only the opening movement. During natural chewing the degree of activity was greatest with bread (bread 196 ± 6 μV apple 168 ± 6 μV) during unilateral chewing the largest maximal mean voltage was found on the ipsilateral side (ipsilat. 179 ± 8 μV contralat 139 ± 8 μV) On the average the right and left anterior temporal muscles were innervated symmetrically during natural chewing during unilateral chewing the activity occurred at first ipsilaterally (time dispersal 10–20 msec)

The findings from the other elevator muscles and from the depressor muscles are given as the average of the degree and the time course of the activity in the right and left muscle during natural (apple, bread) and unilateral (ipsilateral and contralateral, gum) chewing differences between the right and left sides are described separately

Elevator muscles During natural chewing the *posterior part of the temporal muscle* was activated simultaneously with but 25 per cent less than the anterior part, the degree of activity was largest during chewing of bread During unilateral chewing the posterior parts of the temporal muscle showed a larger delay (30–40 msec) between the ipsilateral and contralateral action than the anterior parts the degree of activity was the same on the chewing and balancing side *The masseter muscle* was activated in time with the reference muscle during natural chewing and had most activity during chewing of bread (bread 173 ± 7 μV apple 146 ± 6 μV) during unilateral chewing the masseter muscle showed most activity on the side of chewing (ipsilat 137 ± 6 μV contralat 63 ± 5 μV) but in contrast to the temporal muscles the contralateral muscle was activated before the ipsilateral Among the elevators *the internal pterygoid muscle* was activated first during chewing of apple and bread, the pattern of activity during unilateral chewing was similar to that of the masseter i e the largest activity on the ipsilateral side but the earliest onset of activity contralaterally (ipsilat 203 ± 7 μV contralat. 126 ± 8 μV, time difference 30–60 msec)

Depressor muscles *The external pterygoid muscle* showed the strongest activity during the opening movement (primary activity) but this muscle was active simultaneously with the elevators as well (secondary activity) During natural chewing the primary activity was the same with apple and bread the secondary activity was 30 per cent stronger during chewing of bread as compared to apple During unilateral chewing the primary activity was greatest in the contralateral muscle (contralat 167 ± 5 μV ipsilat 145 ± 5 μV) the secondary action was greatest ipsilaterally (79 ± 4 μV as compared to 55 ± 3 μV contralaterally) *The digastric muscle* was activated in time with the maximal activity in the reference muscle The association between the time of maximal activity in the anterior temporal muscle (elevator) and the onset of activity in the digastric muscle (depressor) was present in all subjects the time to onset of activity in the digastric increased with the time to maximal activity in the anterior temporal muscle (coefficient of correlation $r = +0\,40$ $0\,01 < p < 0\,05$) During unilateral chewing the digastric muscle was most active on the balancing side (contralat 149 ± 6 μV ipsilat 120 ± 5 μV) Also *the mylohyoid muscle* was most active on the balancing

side (contralat 141 ± 6 μV, ipsilat 117 ± 6 μV) It reached maximum before the digastric muscle, during natural chewing the period of strong activity in the mylohyoid muscle occurred before this period had ceased in the right anterior temporal muscle

Orbicularis oris muscles Recordings from the upper and lower lip on the right side showed the largest activity during the opening movement (primary activity), as the external pterygoid the muscles of the lips were also activated simultaneously with the elevators (secondary activity) The primary activity in the lower lip (natural chew 139 ± 8 μV unilat 119 ± 9 μV) was twice that in the upper one (natural chew 64 ± 4 μV unilat. 40 ± 3 μV) The secondary activity was the same in both lips (3–6 times the activity recorded with the mandible at rest)

Variation in the individual subject The variation in the degree and the time course of the activity in the reference muscle during two experiments within the same session (in terms of the standard deviation of the difference) was 4–7 times larger than the variation caused solely by errors of measuring The variation increased by 30–50 per cent when the comparison comprised experiments performed on different days i e with a change in the position of the electrode The difference between the maximal mean voltage in the right and left of the four pairs of elevators was largest in the masseter muscles (60–70 per cent larger than in the temporal and internal pterygoid muscle) Only in the case of the external pterygoid muscles (primary activity) was a significant predominance of the muscle on the same side observed both during the chewing of apple and of bread the persisting predominance was most likely caused by the difficult conditions of recording As judged from the degree of activity in the elevators natural chewing was performed consistently on the same side in only one subject.

Tooth contact Contact between the upper and lower incisors was made shortly after the time of maximal activity in the right anterior temporal muscle and broke about 70 msec later than the cessation of the activity in this muscle During natural chewing contact on the incisors was delayed relative to the molars both at the time of make and break. During unilateral chewing the upper and lower teeth made the first contact on the contralateral side i e on the balancing side The delay between the electrical and mechanical activity of muscle (probably 100 msec during chewing) explains why tooth contact outlasted the activity in the right anterior temporal muscle

Swallowing

In contrast to chewing swallowing was characterized by a synergistic activation of all the muscles under study The degree of activity varied widely from subject to subject

Elevator muscles The activity in the right anterior temporal muscle during swallowing of saliva lasted for about one sec and strong activity was present for 700 msec The onset of activity was synchronous in the two parts of the temporal muscle, the internal pterygoid muscles were activated 45 msec before the masseter muscles 40 msec later than the temporal muscle All elevator muscles were less active during swallowing than during chewing The degree of activity during swallowing of apple was from 25 (internal pterygoid m) to 60 per cent (masseter m) larger than during swallowing of saliva

Depressor muscles During swallowing of saliva the external pterygoid and the mylohyoid muscles were activated before (45 and 30 msec respectively) the anterior temporal in the further course of swallowing the three muscles were innervated simultaneously The onset of activity was synchronous in the temporal and digastric muscles but strong activity in the digastric muscle was delayed 150 msec relative to the temporal The degree of activity in the external pterygoid muscle was less during swallowing than during chewing the digastric and mylohyoid muscles showed the largest activity during swallowing During swallowing of apple the maximal mean voltage in the external pterygoid muscle increased by 15 per cent in the digastric and mylohyoid muscles the mean voltage was the same as during swallowing of saliva

Orbicularis oris muscles During swallowing of saliva the activity in the upper lip started at the same time as in the temporal muscle the activity in the lower lip started 60–70 msec earlier and the phase of strong activity occurred 150 msec before this phase in the temporal muscle Most subjects had low activity in the muscles of the lips during swallowing the maximal mean voltage in the lower lip was twice that in the upper one During swallowing of apple the maximal mean voltage increased (100 per cent in the upper 70 per cent in the lower) and the phase of strong activity in both lips occurred earlier than in the temporal muscle the degree of activity was the same during chewing and swallowing of apple

Tooth contact During swallowing of saliva tooth contact occurred constantly in 23 of 36 subjects 7 subjects swallowed without tooth contact and in

6 contact occurred irregularly Subjects with tooth contact during swallowing had more activity in the anterior temporal the masseter and the orbicularis oris muscles than subjects without contact The degree of activity in the other muscles under study during swallowing was the same in subjects with and without tooth contact

Tooth contact was made earlier on the incisors than on the molars this time-difference and the simultaneous activation of the external pterygoid and the elevator muscles seems to indicate that the mandible moves forward and upward from its position at rest during the initial phase of swallowing The delayed make of contact between the molars most likely occurs after a backward movement to the intercuspal position Tooth contact broke simultaneously on the incisors and molars

Coordination in the individual subject The degree of activity in (1) the anterior and posterior temporal and the masseter (2) the mylohyoid and digastric and (3) the orbicularis oris muscles varied during swallowing in the same way (positive correlation) there was no association between the innervation of the three groups of muscles

Postural activity

With the mandible at rest the activity was slight (2–5 per cent) compared to that observed during chewing and swallowing By the elevation of the mandible from its position at rest to the position of earliest tooth contact the activity increased slightly in the anterior and posterior temporal and in the external pterygoid muscles in the remaining muscles the degree of activity was the same in both positions of the mandible

Full effort

The degree of activity in the elevator muscles during *maximal bite* in the intercuspal position represented the largest degree of activity in most subjects (30 of 36)

Experiments during full effort with the mandible in different positions showed (1) that the two parts of the temporal muscle were active only in the intercuspal position (2) the temporal muscle was passive during biting on the incisors (imitating conditions during biting off) and the mandible was fixed by the masseter and the internal and external pterygoid muscles (3) the external pterygoid muscles were innervated strongly in all experi

ments but (in accordance with the direction of their fibres) most intensively during protrusion (4) in the digastric muscles strong activity was only present during maximal opening

THE ACTIVITY IN THE MUSCLES OF MASTICATION AS RELATED TO THE MORPHOLOGY OF THE FACIAL SKELETON

Aside from measurements of the width and the spacing of the dental arches the analysis of the correlation between the action of the muscles and the morphology of the chewing apparatus was confined to vertical and sagittal dimensions

1 A curved cranial base was associated with. (a) late activation of the masseter muscles relative to the right anterior temporal muscle during chewing, especially during unilateral chewing, (b) strong activity in the anterior and posterior temporal and the masseter muscles during swallowing. In addition the action of the masseter muscle during swallowing was correlated to facial prognathism. strong activity was found in subjects with prognathism and anterior inclination of both jaws

2 A curved mandibular base and prognathism and anterior inclination of the mandible was associated with (a) strong activity in the anterior temporal and the masseter muscles during maximal bite in the intercuspal position and (b) a prolonged initial phase of low activity in the mylohyoid muscle during swallowing of saliva.

3 Retroclination of the lower incisors and reduced alveolar, mandibular prognathism was associated with (a) strong activity in the external pterygoid muscle during swallowing and with the mandible at rest, b) strong postural activity in the digastric muscle and (c) strong activity in the lips during swallowing (c did not include the alveolar prognathism of the mandible)

4 A large overbite was associated with (a) strong activity in the posterior temporal muscle during chewing and during maximal bite in the intercuspal position (b) early activation of the external pterygoid and the digastric muscles relative to the right anterior temporal muscle during chewing (c) low activity in the mylohyoid muscle during swallowing and (d) strong activity in the upper lip during chewing (secondary activity)

The degree of activity in the lips during swallowing depended both on the degree of lip insufficiency (morphology) and the time course of the activity in the mylohyoid muscle

SAMMENDRAG

(Danish summary)

Formålet med nærværende arbejde har været (1) at klarlægge betydningen af de fysiske variable (elektrodernes impedans og placering forstærker og registreringsapparatets indgangsimpedans og frekvensområde) ved elektromyografiske undersøgelser af kæbemusklernes funktion (2) at få et kvantitativt udtryk for graden og tidsforløbet af den elektriske aktivitet i lukke åbne- og læbemusklerne hos unge voksne mænd under tygning synkning i hvile og ved maximal vilkårlig innervation (3) at bestemme tidspunktet for indtræden og ophør af tandkontakt under tygning og synkning og (4) at analysere sammenhængen mellem musklernes elektriske aktivitet og formen af basis cranii kæberne og tand og alveolarbuerne

FREMGANGSMÅDE

I en del metodologiske undersøgelser blev den elektriske muskelaktivitet registreret med en otte kanal elektroencefalograf med blækskrivere Yderligere metodologiske undersøgelser samt alle undersøgelser af muskelaktiviteten under naturlig funktion blev foretaget med en tre kanal elektromyograf med fotografisk registrering fra katodestraleoscillografer Elektromyografens ene forstærker blev udskiftet med et apparat til bestemmelse af den elektriske aktivitets numeriske middelamplitude

I statiske forsøg (metodologiske undersøgelser muskelaktiviteten i hvile og ved maximal vilkårlig innervation) blev aktivitetsgraden bestemt ud fra middelamplitudens gennemsnitlige niveau i registreringer foretaget med en papirhastighed på 5 cm/sek

Den elektriske aktivitet i *dynamiske forsøg* (tygning og synkning) karakteriseredes ved (1) tidspunktet for aktivitetens indtræden og ophør bestemt på elektromyogrammerne (2) den periode af kontraktionen hvor den elektriske aktivitet var størst fastlagt ved hjælp af middelamplituderegistreringen og (3) den maximale middelamplitude I de dynamiske forsøg var registreringshastigheden 20 cm/sek.

Elektrodeimpedansen Overfladeelektrodernes impedans var afhængig af individuelle forskelle mellem elektroderne, af placeringen og den forudgående behandling af huden Elektroder på m temporalis havde impedanser svarende til og højere end de impedanser, der måltes for koncentriske nåleelektroder, elektroimpedansen ved placering på m masseter svarede til de impedanser der måltes mellem to jordelektroder, impedansen mellem to overfladeelektroder kunne reduceres op til 70 % ved gentagen rensning af huden med æter

Differensforstærkningen Aktivitetsgraden i den enkelte muskel bestemmes som spændingsforskellen mellem afledningselektrodens to afledningssteder kanylen og inderlederen i den koncentriske nåleelektrode og de to elektroder der samlet betegnes som en bipolær eller unipolær overfladeelektrode forstærkningen af en fællesspænding på de to afledningssteder (f eks forårsaget af aktivitet i nabomuskler) bør være mindst mulig Med overfladeelektroder var differensforstærkningen stor i forhold til forstærkningen af en fællesspænding både ved benyttelse af elektroencefalograf og elektromyograf Ved afledning med koncentriske nåleelektroder og registrering med elektroencefalograf (lav indgangsimpedans) var differensforstærkningen særlig ringe i frekvensområdet 5–50 Hz Nåleelektroder giver derfor kun en lokaliseret afledning når de benyttes i forbindelse med en forstærker med høj indgangsimpedans

Forstærkerens indgangsimpedans og frekvensområde Når den elektriske aktivitet i m biceps brachii blev registreret med elektroencefalograf var middelamplituden 25 % (overfladeelektroder) og 60 % (nåleelektroder) mindre end ved registrering med elektromyograf Af denne formindskelse skyldtes 10–15 % spændingsdeling mellem elektrode og elektroencefalograf på grund af sidstnævntes lave indgangsimpedans Den resterende del af reduktionen var en følge af elektroencefalografens lave øvre frekvensgrænse

Bipolære og unipolære overfladeelektroder Der afledtes aktivitet fra omkringliggende muskler når den ene elektrode var placeret i nogen afstand fra den muskel der skulle undersøges (»unipolære« placering) Ved bipolær afledning med en elektrodeafstand der var lille i forhold til muskelens størrelse opfangedes aktivitet fra andre muskler identisk af de to elektroder og registreredes derfor ikke

Endelig viste afledning med multielektrode fra m biceps brachii at aktivitetsgraden mellem to tæt beliggende steder (2 mm) kunne variere stærkt

nyttes som udtryk for en forskel i muskelfunktionen

DEN ELEKTRISKE AKTIVITET I KÆBEMUSKULATUREN UNDER NATURLIG FUNKTION

Undersøgelsen af kæbemusklernes naturlige funktion og sammenhængen mellem denne og tyggeapparatets form blev foretaget pa 36 mandlige tand lægestuderende Aktiviteten i de forskellige muskler samt tidspunktet for indtræden og ophør af kontakt mellem incisiverne (36 personer) og molarer ne (10 personer) kunne sammenholdes ved i alle forsøg at aflede fra den samme muskel (referencemuskelen) pa elektromyografens ene kanal

Muskelaktiviteten under tygning

Undersøgelserne af tygning omfattede naturlig tygning af brød og æble og unilateral tygning af tyggegummi Den elektriske aktivitet i reference muskelen (højre m temporalis ant) havde i den enkelte tyggebevægelse en varighed pa 300 msek hvoraf perioden med kraftig aktivitet udgjorde min dre end 200 msek Den totale varighed af den enkelte tyggebevægelse var 580–680 msek

Lukkemusklerne Under naturlig tygning innerveredes den anteriore og posteriore del af m temporalis samt m masseter samtidigt m pterygoideus internus viste aktivitet før de øvrige lukkemuskler og havde den længste va righed af den kraftige aktivitet Ved unilateral tygning fandtes for alle lukke muskler (m temporalis post undtaget) den største aktivitet pa tyggesiden medens m temporalis to dele innerveredes først på tyggesiden (ipsilateralt) indtradte aktiviteten i m masseter og m pterygoideus internus først på ba lancesiden (kontralateralt) Aktiviteten i lukkemusklerne nåede maximum kort før indtræden af tandkontakt og den ophørte i den første tredjedel af kontaktintervallet

Åbnemusklerne Under tygning viste m pterygoideus externus den kraftig ste aktivitet under abnebevægelsen (primær aktivitet) men denne muskel var også aktiv sammen med lukkemusklerne (sekundær aktivitet) Aktiviteten i m digastricus begyndte samtidig med at aktiviteten i referencemuskelen nåede sit *maximum* Dette træk i koordinationen af m temporalis anterior (lukkemuskel) og m digastricus (åbnemuskel) fandtes hos alle personer JO

længere tid aktiviteten i m temporalis var om at nå sit maximum desto senere aktiveredes m digastricus Aktiviteten i m mylohyoideus nåede maximum før end i m digastricus og under naturlig tygning begyndte perioden med kraftig aktivitet i m mylohyoideus før samme periode var afsluttet i referencemuskelen Ved unilateral tygning viste alle åbnemuskler størst aktivitet på balancesiden (kontralateralt) I forhold til tandkontaktintervallet under tygning aktiveredes m mylohyoideus før og m digastricus samtidig med kontaktens indtræden medens den primære aktivitet i m pterygoideus externus først indtrådte kort før ophør af tandkontakt

Læbemusklerne Under tygning viste *m orbicularis oris* den kraftigste aktivitet svarende til åbnebevægelsen (primær aktivitet) ligesom i m pterygoideus externus fandtes i læberne en aktivitetsfase samtidig med lukkemuskulaturen (sekundær aktivitet) Den primære aktivitet i underlæben var dobbelt så kraftig som i overlæben den sekundære aktivitet var af samme størrelsesorden i begge læber

Tandkontakt Kontakt mellem over og underkæbens incisiver indtrådte kort efter aktivitetsmaximet i referencemuskelen og ophørte ca 70 msek efter ophøret af aktiviteten i denne muskel Under naturlig tygning var tandkontakt i incisivregionen forsinket i forhold til molarregionerne både med hensyn til indtræden og ophør Ved unilateral tygning mødtes over- og underkæbens tænder først kontralateralt dvs på balancesiden Forsinkelsen mellem den elektriske og den mekaniske muskelaktivitet kan forklare hvorfor tandkontakt ophørte senere end den elektriske aktivitet i referencemuskelen (m temp ant)

Muskelaktiviteten under synkning

I modsætning til tygning viste synkning en synergistisk aktivering af alle de undersøgte muskler Aktivitetsgraden i de forskellige muskler varierede stærkt fra person til person

Lukkemusklerne Aktiviteten i højre m temporalis anterior under synkning af spyt varede i alt ca 1 sek, og kraftig aktivitet var til stede i 700 msek I m pterygoideus internus indtrådte aktiviteten 45 msek tidligere, i masseter 40 msek senere end i m temporalis to dele, den kraftige aktivitet i lukkemusklerne forekom samtidig

Åbnemusklerne Under synkning af spyt begyndte aktiviteten i m pterygoideus externus og m mylohyoideus henholdsvis 45 og 30 msek tidligere end i m temporalis anterior det videre aktivitetsforløb i disse muskler var sam

menfaldende Aktiviteten i m digastricus og m temporalis begyndte samtidig men den kraftige kontraktionsfase var 150 msek forsinket i m digastricus i forhold til m temporalis

Lukkemusklerne samt m pterygoideus externus viste størst aktivitet under tygning medens m digastricus og m mylohyoideus viste størst aktivitet under synkning

Læbemusklerne Ved synkning af spyt begyndte aktiviteten i overlæben samtidig med referencemuskelen aktiviteten i underlæben begyndte 60–70 msek før Den kraftige aktivitet i underlæben indtradte 150 msek før den kraftige aktivitet i m temporalis anterior De fleste forsøgspersoner havde lav aktivitet i læbemusklerne under synkning aktiviteten i underlæben var dobbelt så kraftig som i overlæben

Tandkontakt under synkning af spyt fandtes regelmæssigt hos 23 af 36 personer hos 7 personer forløb synkningen altid uden tandkontakt og hos 6 personer optradte tandkontakt lejlighedsvis Personer med tandkontakt havde kraftigere aktivitet i m temporalis anterior m masseter og i over og underlæbens muskler end personer uden tandkontakt de øvrige muskler viste ingen forskelle i aktivitetsgraden mellem personer med og uden tandkontakt under synkning

Tandkontakt indtradte før pa incisiverne end pa molarerne denne tidsforskel og det forhold at m pterygoideus externus aktiveredes samtidig med lukkemusklerne tyder pa at underkæben i synkningens initiale del føres fremad og opad den forsinkede molarkontakt oprettes formentlig efter en bevægelse bagud til den habituelle okklusionsstilling Tandkontakt ophørte samtidigt pa incisiver og molarer

Muskelkoordinationen hos samme person Aktiviteten i henholdsvis (1) m temporalis anterior og posterior og m masseter (2) m mylohyoideus og m digastricus og (3) over og underkæbens muskler varierede under synkning i samme retning (positiv korrelation) der var ingen sammenhæng mellem innervationen af disse 3 muskelgrupper

Muskelaktiviteten i hvile

Med underkæben i hvile var aktiviteten ringe (2–5 %) i forhold til aktiviteten under tygning og synkning Når underkæben blev hævet fra hvilestilling til første tandkontakt øgedes innervationen af m temporalis anterior og posterior samt af m pterygoideus externus I de øvrige muskler var aktivitetsgraden i de to stillinger den samme

Aktiviteten ved maximal vilkårlig innervation

Aktiviteten i lukkemuskulaturen ved maximalt sammenbid i den habituelle okklusionsstilling repræsenterede hos størstedelen af forsøgspersonerne den højeste grad af aktivitet

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SAMMENHÆNGEN MELLEM KÆBEMUSKLERNES FUNKTION OG ANSIGTSFORMEN

Udover tandbuebredden og tændernes pladsforhold var analysen af sammenhængen mellem tyggeapparatets funktion og form begrænset til ansigtets sagittale og vertikale dimensioner

1 Ved stærk bøjning af basis cranii fandtes stærk aktivitet i m temporalis anterior og posterior og i m masseter under synkning

2 Ved stærk bøjning af basis mandibulae samt prognati og anterior inklination af underlæben fandtes (a) stærk aktivitet i m temporalis anterior og m masseter ved maximalt sammenbid i den habituelle okklusionsstilling og (b) en lang indledende fase med svag aktivitet i m mylohyoideus under synkning af spyt

3 Ved stort vertikalt overbid (dybt bid) fandtes (a) kraftig aktivitet i m temporalis posterior under tygning og ved maximalt sammenbid i den habituelle okklusionsstilling og (b) tidlig aktivering af m digastricus og m pterygoideus externus under tygning (i forhold til m temporalis anterior dex)

Endelig er det påvist at læbeaktiviteten under synkning både kan være udtryk for en tilpasning af de morfologiske forhold (graden af læbeinsufficiens) og til andre funktionelle forhold (tidsforløbet af aktiviteten i m mylohyoideus)

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APPENDIX

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Tables I–XVI Average electrical activity in the muscles of mastication during mastication and swallowing

Table I

Average electrical activity in the right anterior temporal muscle during natural chewing of apple and bread and during unilateral (right and left sided) chewing of chewing gum
All figures were referred to the onset of activity
(36 subjects 16 experiments during each type of chewing)

Parameters	Apple		Bread		Right side (ipsilateral)		Left side (contralateral)		
	M±SE	SD	M±SE	SD	M±SE	SD	M±SE	SD	n[1]
Tot. dur. of act. msec	286±8	38	297±7	41	290±8	44	291±7	43	36
Tot. dur. of chew cycle msec.	588±9	51	632±10	57	675±9	50	676±9	53	36
Time to 50% MV_{m} msec	106±5	31	105±5	31	136±4	25	135±5	30	36
Time to MV_{m} msec	210±6	37	213±6	36	209±5	29	210±6	34	36
Time to 50% decline from MV_{m} msec	277±6	36	288±7	39	275±7	38	270±7	39	36
MV_{m} μV	168±6	37	196±6	39	179±8	47	139±8	45	36

M average SE standard error SD standard deviation MV mean voltage
(1) To calculate the total average the data from 16 experiments were averaged in each subject.

Table II

Average electrical activity in the left anterior temporal muscle during natural chewing of apple and bread and during unilateral (right and left sided) chewing of chewing gum
All figures were referred to the onset of activity in the right anterior temporal muscle (36 subjects 1 experiment during each type of chewing)

Parameters	Apple		Bread		Left side (ipsilateral)		Right side (contralateral)		
	M±SE	SD	M±SE	SD	M±SE	SD	M±SE	SD	n
Onset of act. msec	−6±5	27	—(1)	—	−18±6	33	10±5	33	36
Cessation of act. msec	281±9	50	—(1)	—	249±7	42	280±9	52	36
Time to 50% MV_{m} msec	88±7	43	—(1)	—	105±8	47	137±7	43	36
Time to MV_{m} msec	197±8	44	—(1)	—	180±7	43	212±8	45	36
Time to 50% decline from MV_{m} msec	271±8	49	—(1)	—	239±7	39	269±8	48	36
MV_{m} μV	160±7	44	177±9	53	153±8	50	136±9	54	36

M average negative values indicate that the activity occurred before the reference point (time zero)
SE standard error SD standard deviation MV mean voltage
(1) The variance analysis showed a random variation between chewing of apple and bread to calculate the total average the data from the two types of chewing were averaged in each subject.

Tables III–VIII

Average electrical activity in the elevator (except in the anterior temporal) and the depressor muscles during natural chewing of apple and bread and unilateral (ipsilateral and contralateral) chewing of chewing gum. During natural chewing all figures were referred to the onset of activity in the right anterior temporal muscle; during unilateral chewing to an intermediate reference point half way between the ipsilateral and contralateral onset of activity in the right anterior temporal muscle (for definition see p 82 and Fig 30). The data are given as the average of the activity in the right and the left muscle of a pair (36 subjects, 1 experiment on each muscle during each type of chewing)

Table III Posterior temporal muscles

Parameters	Apple			Bread			Ipsilateral			Contralateral		
	M±SE	SD	n	M±SE	SD	n	M±SE	SD	n	M±SE	SD	n
Onset of act msec	0±5	44	72	−2±6	50	72	6±7	40	36	45±7	39	36
Cessation of act msec	277±7	55	72	291±7	60	72	258±7	40	36	285±7	42	36
Time to 50 / MV_m msec	108±7	56	72	—[1]	—	—	105±6	52	72	134±7	55	72
Time to MV_m msec	215±6	48	72	—[1]	—	—	197±5	46	72	208±6	48	72
Time to 50 / decline from MV_m msec	275±6	49	72	—[1]	—	—	252±7	38	36	272±7	38	36
MV_m µV	110±5	46	72	141±6	54	72	94±5	45	72	99±5	45	72

Table IV Masseter muscles

Parameters	Apple			Bread			Ipsilateral			Contralateral		
	M±SE	SD	n	M±SE	SD	n	M±SE	SD	n	M±SE	SD	n
Onset of act msec	−4±4	30	72	—[1]	—	—	17±5	27	36	-10±6	36	36
Cessation of act msec	268±6	51	72	288±7	55	72	272±6	49	72	251±7	56	72
Time to 50 / MV_m msec	85±6	36	36	—[1]	—	—	133±5	31	36	85±8	49	36
Time to MV_m msec	192±6	52	72	—[1]	—	—	199±5	40	72	179±7	60	72
Time to 50 / decline from MV_m msec	257±6	51	72	271±7	59	72	257±5	44	72	241±7	53	72
MV_m µV	143±6	50	72	173±7	[illegible]	72	137±6	54	72	63±5	38	72

e V Internal pterygoid muscles

Parameters	Apple			Bread			Ipsilateral			Contralateral		
	M±SE	SD	n	M±SE	SD	n	M±SE	SD	n	M±SE	SD	n
t of act ec	−37±6	51	72	–(1)	–	–	−14±10	69	36	−40±8	47	36
ation of act ec	316±12	73	36	–(1)	–	–	333±12	72	36	285±13	76	36
to 50 / MV_m ec	33±7	58	72	–(1)	–	–	81±11	68	36	17±9	54	36
to MV_m × ec	171±7	63	72	–(1)	–	–	203±10	57	36	139±12	71	36
to 50 / decline from V_m × msec	285±9	72	72	292±9	72	72	297±8	66	72	250±10	83	72
	189±6	46	72	200±5	44	72	203±7	43	36	126±8	49	36

e VI External pterygoid muscles

Parameters	Apple			Bread			Ipsilateral			Contralateral		
	M±SE	SD	n	M±SE	SD	n	M±SE	SD	n	M±SE	SD	n
ary activity												
t of act ec	300± 6	53	72	313± 7	59	72	331±10	85	72	350± 9	74	72
ation of act sec	635±14	85	36	694±15	90	36	706±14	119	72	756±15	127	72
e to 50 MV_m sec	360± 7	55	72	–(1)	–	–	389±10	88	72	414± 9	76	72
e to MV_m sec	467± 9	72	72	–(1)	–	–	535±13	79	36	–(1)	–	–
e to 50 ne from MV sec	598±10	85	72	661±11	96	72	672±17	100	36	708±16	96	36
	186± 6	46	72	199± 5	41	72	145± 5	45	72	167± 5	44	72
ndary activity												
t of act sec	32± 7	57	72	38± 5	46	72	−3± 6	37	36	30± 7	38	36
ation of act sec	293 7	43	36	–(1)	–	–	320±10	58	36	–(1)	–	–
e to MV_m sec	187− 7	38	36	–(1)	–	–	216± 9	54	36	195± 8	45	36
	64 4	34	72	89± 4	34	72	79± 4	37	72	55± 3	28	72

Table VII Digastric muscles

Parameters	Apple M±SE	SD	n	Bread M±SE	SD	n	Ipsilateral M±SE	SD	n	Contralateral M±SE	SD	n
Onset of act msec	217±10	57	36	237±8	48	36	242±12	69	36	–(2)	–	–
Cessation of act msec	615±11	67	36	682±15	90	36	679±18	109	36	745±22	129	36
Time to 50 / MV_m msec	303±9	73	72	324±9	74	72	342±14	83	36	372±15	89	36
Time to MV_m msec	445±10	63	36	478±11	68	36	502±16	96	36	548±16	98	36
Time to 50 / decline from MV_m msec	575±10	63	36	630±14	83	36	637±18	109	36	689±18	108	36
MV_m µV	167±5	41	72	–(1)	–	–	120±5	32	36	149±[illegible]	36	36

Table VIII Mylohyoid muscles

Parameters	Apple M±SE	SD	n	Bread M±SE	SD	n	Ipsilateral M±SE	SD	n	Contralateral M±SE	SD	n
Onset of act msec	200±9	75	72	217±9	75	72	224±14	85	36	262±14	83	36
Cessation of act msec	621±17	140	72	677±19	162	72	655±18	107	36	732±22	133	36
Time to 50 / MV_m msec	279±8	69	72	–(1)	–	–	325±17	102	36	356±16	96	36
Time to MV_m msec	392±12	99	72	441±14	120	72	458±14	117	72	509±14	119	72
Time to 50 / decline from MV_m msec	557±20	116	36	602±23	135	36	594±14	116	72	658±16	135	72
MV_m µV	169±5	57	72	–(1)	–	–	117±6	49	72	141±6	54	72

M average negative values indicate that the activity occurred before the reference point (time zero)
SE standard error SD standard deviation MV mean voltage
n = 36 the variance analysis showed a random variation between the right and the left muscle to calculate the total average the data from the two muscles were averaged in each subject
n = 72 the variance analysis showed a significant variation between the right and the left muscle or a significant interaction between muscles and subjects or types of chewing to calculate the total average the data from the right and the left muscle were used as single observations
(1) and (2) The variance analysis showed a random variation between chewing of apple and bread (1) and between ipsilateral and contralateral chewing (2) to calculate the total average the data from the two types of natural chewing and from the two types of unilateral chewing were averaged in each subject

Tables IX–X

Average electrical activity in the muscles of the upper and lower lip during natural chewing of apple and bread and unilateral (right and left sided) chewing of chewing gum During natural chewing all figures were referred to the onset of activity in the right anterior temporal muscle during unilateral chewing to an intermediate reference point half way between the ipsilateral and contralateral onset of activity in the right anterior temporal muscle (for definition see p 82 and Fig 30) (36 subjects 1 experiment on each lip during each type of chewing)

Table IX Upper lip

Parameters	Apple			Bread			Unilateral [1] (right and left sided chew)		
	M±SE	SD	n	M±SE	SD	n	M±SE	SD	n
Primary activity									
Onset of act msec	315±10	58	36	333±10	58	36	364± 9	54	36
Cessation of act msec	612±11	63	36	–[2]	–	–	680±13	76	36
Time to 50 % MV_m msec	381±12	71	36	399±10	62	36	444± 9	53	36
Time to MV_{mx} msec	464±11	67	36	508±12	71	36	549±11	[illegible]	36
Time to 50 % decline from MV_{mx} msec	568±12	70	36	611±13	76	36	653±12	71	36
MV_m µV	64± 4	26	36	–[2]	–	–	40± 3	15	36
Secondary activity									
Onset of act msec	51± 7	38	36	–[2]	–	–	64± 5	31	36
Cessation of act msec	299± 7	41	36	–[2]	–	–	299±10	61	36
Time to 50 % MV_m msec	90± 7	42	36	–[2]	–	–	106± 6	37	36
Time to MV_m msec	190± 8	49	36	–[2]	–	–	194± 6	38	36
Time to 50 % decline from MV_{mx} msec	278± 8	47	36	–[2]	–	–	270± 8	49	36
MV_{mx} µV	40± 3	20	36	–[2]	–	–	20±1	8	36

Table X Lower lip

Parameters	Apple			Bread			Unilateral [1] (right and left sided chew)		
	M±SE	SD	n	M±SE	SD	n	M±SE	SD	n
Primary activity									
Onset of act msec	319±12	69	36	331±12	73	36	352±13	76	36
Cessation of act msec	609±14	86	36	–[2]	–	–	650±15	93	36
Time to 50 % MV_m msec	381±12	71	36	396±11	65	36	433±11	66	36
Time to MV_m msec	480±13	[illegible]	36	488±12	70	36	531±12	72	36
Time to 50 % decline from MV_m msec	583±15	87	36	593±15	[illegible]	36	624±14	84	36
MV_m, µV	139± 8	48	36	–[2]	–	–	119± 9	51	36
Secondary activity									
Onset of act msec	47±13	74	36	–( )	–	–	27±15	87	36
Cessation of act msec	325±11	67	36	–[2]	–	–	343±13	79	36
Plateau of MV µV	32± 3	[illegible]	36	–( )	–	–	22± 2	13	36

M average SE standard error SD standard deviation MV mean voltage

[1] The variance analysis showed a random variation between right and left sided chewing to calculate the total average the data from the two types of chewing were averaged in each subject

( ) The variance analysis showed a random variation between chewing of apple and bread to calculate the total average the data from the two types of chewing were averaged in each subject

Tables XI and XII

Average electrical activity (36 subjects) in the reference muscles (right anterior temporal and right mylohyoid m.) and the corresponding muscles on the left side during swallowing. The recordings were obtained simultaneously from each pair of muscles and all figures were referred to the onset of activity in the muscle on the right side

Table XI

	Right anterior temporal muscle					Right mylohyoid muscle				
	Saliva		Apple			Saliva		Apple		
	M±SE	SD	M±SE	SD	n[1]	M±SE	SD	M±SE	SD	n[2]
Tot dur of act msec	1060±40	240	925±35	220	36	1125±30	175	1050±30	185	36
Time to 50% MV$_m$ msec	220±20	130	180±15	75	36	265±20	125	170±15	95	36
Time to MV msec	555±25	155	435±15	105	36	550±30	175	410±25	150	36
Time to 50 decline from MV$_m$ msec	895±35	200	760±35	195	36	820±35	205	755±35	195	36
MV µV	84±10	60	113±11	67	36	211±8	50	227±9	52	36

Table XII

	Left anterior temporal muscle					Left mylohyoid muscle				
	Saliva		Apple			Saliva		Apple		
Parameters	M±SE	SD	M±SE	SD	n[2]	M±SE	SD	M±SE	SD	n[3]
Onset of act msec	2±4	25	–[4]	–	36	1±3	20	–[4]	–	36
Cessation of act msec	1025 50	305	850±50	285	36	1115±35	200	–[4]	–	36
Time to 50 MV msec	200±15	95	–[4]	–	36	295±35	205	170±25	140	36
Time to MV msec	480±35	210	425±30	190	36	560±35	210	430±40	255	36
Time to 50 decline from MV msec	845±50	290	730±50	310	36	855±35	205	–[1]	–	36
MV µV	87 11	65	–[4]	–	36	217±9	55	–[4]	–	36

M average SE standard error SD standard deviation MV mean voltage

[1] Average of 16 experiments on each subject

[2] Average of 7 experiments on each subject

[3] 1 experiment on each subject

[4] The variance analysis showed a random variation between swallowing of saliva and of apple in calculate the total average the data from the two types of swallowing were averaged in each subject

Table XIII

Average time dispersal between the onset of activity in the test muscles and the right anterior temporal muscle (EMG 2 cf Fig 25) *during swallowing of saliva and apple* (other time and amplitude data see Tables XIV XV XVI) In the elevator and depressor muscles the time dispersal represents the average of the right and left muscle (one experiment on each muscle during each type of swallowing)

Test muscle	Number of subjects (n)	Swallowing saliva M±SE msec	Swallowing saliva t	Swallowing apple M±SE msec	Swallowing apple t
Posterior temporal	36	15±8	1.9	−7±5	1.4
	32	13±8	1.7	−5±6	0.8
	19	13±12	1.1	8±7	1.1
Masseter	36	41±15	2.7	25±10	2.5
	32	44±12	3.7*	21±8	2.6*
	19	67±16	4.2**	31±10	3.1
Internal pterygoid	36	−43±15	2.9*	−51±16	3.2*
	32	−39±13	3.0	−46±13	3.5*
	19	−47±18	2.6	−24±10	2.4
External pterygoid	36	−45±13	3.5	1±11	0.1
	32	−47±10	4.7*	2±10	0.2
	19	−37±12	3.1*	−4±11	0.4
Digastric	36	4±17	0.2	51±19	2.7
	32	15±13	1.2	58±16	3.6*
	19	31±18	1.7	47±18	2.6
Mylohyoid	36	−32±10	3.2*	11±10	1.1
	32	−27±9	3.0**	12±12	1.0
	19	−12±8	1.5	8±17	0.5
Upper lip	36	−10±28	0.4	−35±22	1.6
	32	−4±30	0.1	−37±23	1.6
	19	30±39	0.8	−62±36	1.7
Lower lip	36	−66±43	2.9	−42±20	2.1*
	32	−73±25	3.2	−48±22	2.2
	19	−59±28	2.0	−66±33	2.0*

M average time dispersal SE standard error the significance of the time dispersal was tested by calculating $t = \frac{M}{SE}$ thus * 0.01 < p < 0.05 ** 0.001 < p < 0.01 *** p < 0.001

36 subjects all subjects examined

32 subjects 4 subjects with consistently low activity in the right anterior temporal muscle were omitted

19 subjects subjects with a maximal mean voltage of 50 µV or more in the right anterior temporal muscle

Negative values indicate that the muscle was innervated before the onset of activity in the right anterior temporal muscle

Tables XIV and XV

Average electrical activity in the elevator (except in the anterior temporal) and the depressor muscles during swallowing of saliva and apple. All figures were referred to the onset of activity in the right anterior temporal muscles (time zero) and data are given as the average of the activity in the right and the left muscle of a pair (36 subjects, 1 experiment on each muscle during each type of swallowing)

Table XIV

Elevator muscles	Posterior temporal						Masseter						Internal pterygoid					
	Saliva			Apple			Saliva			Apple			Saliva			Apple		
Parameters[1]	M ± SE	SD	n	M ± SE	SD	n	M ± SE	SD	n	M ± SE	SD	n	M ± SE	SD	n	M ± SE	SD	n
Cessation of act., msec	1005 ± 55	325	36	875 ± 50	290	36	1020 ± 40	240	36	955 ± 40	235	36	1040 ± 30	180	36	–[2]	–	–
Time to 50 % MV_{max}, msec	215 ± 25	150	36	145 ± 20	115	36	255 ± 30	170	36	185 ± 15	95	36	215 ± 25	195	72	115 ± 20	180	72
Time to MV_{max}, msec	565 ± 30	235	72	465 ± 25	220	72	525 ± 30	175	36	415 ± 20	130	36	530 ± 40	240	36	430 ± 35	215	36
Time to 50 % decline from MV_{max}, msec	890 ± 35	315	72	750 ± 35	295	72	820 ± 40	240	36	695 ± 35	205	36	800 ± 30	190	36	–[2]	–	–
MV_{max}, µV	64 ± 6	50	72	75 ± 7	60	72	60 ± 9	56	36	97 ± 12	69	36	142 ± 6	53	72	178 ± 7	60	72

Table XV

Depressor muscles	External pterygoid						Digastric						Mylohyoid					
	Saliva			Apple			Saliva			Apple			Saliva			Apple		
Parameters[1]	M ± SE	SD	n	M ± SE	SD	n	M ± SE	SD	n	M ± SE	SD	n	M ± SE	SD	n	M ± SE	SD	n
Cessation of act., msec	1090 ± 50	295	36	935 ± 45	255	36	1205 ± 50	295	36	1070 ± 35	225	36	1115 ± 40	230	36	1095 ± 50	295	36
Time to 50 % MV_{max}, msec	195 ± 20	180	72	200 ± 20	185	72	385 ± 40	230	36	250 ± 30	165	36	260 ± 25	210	72	215 ± 30	240	72
Time to MV_{max}, msec	575 ± 30	235	72	485 ± 30	240	72	595 ± 40	245	36	475 ± 35	210	36	555 ± 40	230	36	430 ± 40	250	36
Time to 50 % decline from MV_{max}, msec	880 ± 45	260	36	745 ± 35	200	36	895 ± 45	275	36	820 ± 35	210	36	830 ± 40	240	36	825 ± 45	285	36
MV_{max}, µV	69 ± 4	27	36	98 ± 8	46	36	220 ± 8	45	36	–[2]	–	–	204 ± 10	57	36	220 ± 8	47	36

M average, SE standard error, SD standard deviation, MV mean voltage

n = 36 the variance analysis showed a random variation between the right and the left muscle; to calculate the total average the data from the two muscles were averaged in each subject

n = 72 the variance analysis showed a significant variation between the right and the left muscle or a significant interaction between muscles and subjects or types of swallowing; to calculate the total average the data from the right and the left muscle were used as single observations

[1] Onset of activity see Table XIII

[2] The variance analysis showed a random variation between swallowing of saliva and of apple; to calculate the total average the data from the two types of swallowing were averaged in each subject

Table XVI

Average electrical activity in the muscles of the upper and lower lip during swallowing of saliva and apple. All figures were referred to the onset of activity in the right anterior temporal muscle (36 subjects, 1 experiment on each lip during each type of swallowing)

Parameters[1]	Upper lip						Lower lip					
	Saliva			Apple			Saliva			Apple		
	M±SE	SD	n	M±SE	SD	n	M±SE	SD	n	M±SE	SD	n
Cessation of act msec	955±60	350	36	850±40	240	36	975±55	335	36	840±50	300	36
Time to 50 MV msec	120±30	190	36	75±20	120	36	120±30	190	36	60±20	120	36
Time to MV msec	405±50	305	36	300±25	150	36	390±45	280	36	285±25	160	36
Time to 50 decline from MV msec	735±65	400	36	590±40	240	36	645±65	395	36	520±45	275	36
MV µV	36±5	32	36	70±9	57	36	75±9	56	36	127±11	65	36

M average, SE standard error, SD standard deviation, MV mean voltage
[1] Onset of activity see Table XIII